Free fatty acid receptor (FFA) and lipid receptor (GPR119) signalling mediates nutrient-sensing in mouse intestine

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Free fatty acid receptor (FFA) and lipid receptor (GPR119) signalling mediates nutrient-sensing in mouse intestine

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Abstract

Medium and long chain fatty acids exhibit affinity for free-fatty acid receptor 1 (FFA1) and 4 (FFA4). FFA1 and FFA4 are expressed in the pancreas with a third lipid G-protein coupled receptor, GPR119, and all three GPCRs are also highly expressed by gastrointestinal (GI) enteroendocrine L cells, which release glucagon-like peptide 1 (GLP-1) and peptide YY (PYY). GLP-1 is a stimulant of insulin secretion postprandially, whereas PYY slows GI motility and reduces food intake. The receptor expression patterns raise these receptors' potential as targets for anti-diabetic and anti-obesity therapeutics. This thesis compares the pharmacology and cellular signalling of novel FFA1, FFA4 and GPR119 agonists from AstraZeneca, with that of commercially available agonists in mouse colonic mucosa.

Mucosa was mounted in Ussing chambers, voltage-clamped and the resultant short-circuit current (I_{sc}) was recorded continuously, as a measurement of vectorial epithelial ion transport *in vitro*. Colonic bead propulsion and charcoal-meal gavage assessments were used to measure lower and upper GI transit *in vivo*, respectively.

FFA1, FFA4 or GPR119 selective agonism potently inhibited mucosal anion secretion via a PYY-Y_1 paracrine mechanism. These anti-secretory responses showed area-specificity and the descending colon was selected for further interrogation. Here, FFA1 and FFA4 agonists displayed low maximal responses, whereas GPR119 agonists exhibited partial efficacy in comparison to PSN632408. All agonists required glucose for activity, minimising the potential for hypoglycaemia, a limitation of current anti-diabetic drugs. Furthermore, dual agonism of FFA1, FFA4 or GPR119 was additive. The FFA1 antagonist, ANT825 revealed endogenous intestinal FFA1 tone, and this was inhibited in the presence of the competitive Y_1 and Y_2 antagonists. FFA4 agonism slowed transit in the colon and increased transit in the small intestine, whereas FFA1 agonism had no effect on transit *in vivo*. As the FFA4 receptor is more broadly expressed in the GI tract compared to FFA1 and GPR119, luminal-restricted GPR119 agonists in combination with FFA1 agonists should be considered as future T2DM therapeutics.

Table of Contents

Table of Figures & Tables

Chapter 1: Introduction

Chapter 2: Methods & Materials

Chapter 3: Results

Chapter 4: Discussion

Publications

Papers

- Tough, I.R., Moodaley, R., and Cox, H.M. (2018). 'Mucosal glucagon-like peptide 1 (GLP-1) responses are mediated by calcitonin gene-related peptide (CGRP) in the mouse colon and both peptide responses are area-specific'. *Neurogastroenterol. Motil*., 30 (1), e13149.

- Moodaley, R., Smith, D.M., Tough, I.R., Schindler, M., and Cox, H.M. (2017) 'Agonism of free fatty acid receptors 1 and 4 generate peptide YY-mediated inhibitory responses in mouse colon'. *Br. J. Pharmacol.*, 174 (23), 4508- 4522.

Published abstracts

- Moodaley, R., Schindler, M., Smith, D.M., and Cox, H.M. (2017) 'Pinolenic acid co-activates free fatty acid receptor 1 and 4 and this signalling reduces GPR119 agonism in mouse colonic mucosa'. E-journal of British Pharmacology Society http://www.pa2online.org.

- Moodaley, R., Smith, D.M., Schindler, M., and Cox, H.M. (2016) 'GPR119 agonist-mediated PYY release from enteroendocrine L cells requires glucose and epithelial Y_1 receptors'. E-journal of British Pharmacology Society http://www.pa2online.org.

Unpublished abstracts

- Moodaley, R., Schindler, M., Smith, D.M., and Cox, H.M. (2017) 'FFA1 and FFA4 agonism in mouse colon is PYY Y_1-mediated and glucose-sensitive.'
Keystone Meeting: Gastrointestinal Control of Metabolism, Copenhagen, Denmark.

Abbreviations

5-HT; serotonin,

AC; Adenylate cyclase,

ACh; acetylcholine,

AgRP; agouti-related peptide,

AH-7614; (4-Methyl-*N*-9*H*-xanthen-9-yl-benzenesulfonamide),

Akt; protein kinase B,

ANOVA; analysis of variance,

ANS; autonomic nervous system,

ap; apical,

ARC; arcuate nucleus,

ATP; adenosine triphosphate,

AQP; aquaporin,

AZ; AstraZeneca,

BIBO3304; (*N*-[(1*R*)1[[[[4[[(Aminocarbonyl)amino]methyl]phenyl]methyl]amino]carbonyl]-4-[(aminoiminomethyl)amino]butyl]-α-phenyl-benzeneacetamide ditrifluoroacetate),

BIIE0246; (*N*-[(1*S*)-4-[(Aminoiminomethyl)amino]-1-[[[2-(3,5-dioxo-1,2-diphenyl-1,2,4-triazolidin-4-yl)ethyl]amino]carbonyl]butyl]-1-[2-[4-(6,11-dihydro-6-oxo-5*H*-dibenz[*b*,*e*]azepin-11-yl)-1-piperazinyl]-2-oxoethyl]-cyclopentaneacetamide),

bl; basolateral,

BMI; body mass index,

BRET; bioluminescence resonance energy transfer,

$[Ca^{2+}]_{i;}$ intracellular calcium,

Ca^{2+}; calcium ion,

cAMP; cyclic adenosine monophosphate,

CART; cocaine and amphetamine-related transcript,

CB1; cannabinoid receptor 1,

CB2; cannabinoid receptor 2,

CCK; cholecystokinin,

cDNA; complementary deoxyribonucleic acid,

CFTR; cystic fibrosis transmembrane conductance regulator,

CGRP; calcitonin gene-related peptide,

ChAT; choline acetyltransferase,

CHO; Chinese Hamster Ovary

Cl^-; chloride ion,

CNS; central nervous system,

COX-2; cyclooxygenase 2,

Cpd.16; Compound 16,

Cpd.42; Compound 42,

DAG; diacylglycerol,

dH_2O; distilled water,

DHA; docosahexaenoic acid,

DIO; diet-induced obese,

DMSO; dimethyl sulphoxide,

DMPK; drug metabolism and pharmacokinetics,

DMR; dynamic mass redistribution,

DNA; deoxyribonucleic acid,

DPPIV; dipeptidyl peptidase-4,

ECCs; enterochromaffin cells,

EECs; enteroendocrine cells,

e.g.; example,

ENS; enteric nervous system,

Epac; exchange protein directly activated by cAMP,

ER; endoplasmic reticulum,

Ex; exendin,

Ex4; exendin 4,

Ex(9-39); exendin (9-39),

FAAH; fatty acid amide hydrolase,

FAE; follicle-associated epithelium,

FFA, free-fatty acid,

FFA1; free-fatty acid receptor 1,

FFA2; free-fatty acid receptor 2,

FFA3; free-fatty acid receptor 3,

FFA4; free-fatty acid receptor 4,

FLIPR; fluorometric imaging plate reader,

GALT; gut-associated lymphoid tissue,

GCGR; glucagon receptor,

GI; gastrointestinal,

GIP; gastric inhibitory peptide,

GIP-R; gastric inhibitory polypeptide receptor,

GLP-1; glucagon-like peptide 1,

GLP-1R; glucagon like peptide 1 receptor,

GLP-2; glucagon-like peptide 2,

GLP-2R; glucagon-like peptide 2 receptor,

GLUT; glucose transporter,

GPCR; G-protein coupled receptor,

GPR120L; long FFA4 isoform,

GPR120S; short FFA4 isoform,

GRK2; G-protein coupled receptor kinase 2,

GRPP; glicentin-related pancreatic polypeptide,

GSIS; glucose-stimulated insulin secretion,

GSK; GlaxoSmithKline,

GW1100; (4-[5-[(2-ethoxy-5-pyrimidinyl)methyl]-2-[[(4-fluorophenyl)methyl]thio]-4-oxo-1(4H)-pyrimidinyl]-benzoic acid,ethyl ester),

GW9508; (4-[[(3-Phenoxyphenyl)methyl]amino]benzenepropanoic acid),

HbA1c; glycosylated haemoglobin A1c,

HCO_3^-; bicarbonate ion,

HCl; hydrochloride,

HEK; human embryonic kidney cells,

HEPE; hydroxyl-eicosapentaenoic acid,

hERG; human ether-a-go-go related gene;

HFD; high fat diet,

HPMC; hydroxypropyl methylcellulose,

ICC; interstitial cells of Cajal,

icv; Intracerebroventricular,

IKK; I_KB kinase,

i.p.; intraperitoneal injection,

IP; intervening peptide,

IPANs; intrinsic primary afferent neurons,

IP_3; inositol trisphosphate,

I_{sc}; short-circuit current,

JNK; c-Jun NH2-terminal kinase,

JTT; JTT-851,

K^+; potassium ion,

K_{ATP}; ATP-sensitive potassium channels,

KH; Krebs-Henseleit,

LCFA; long-chain fatty acid,

LC-MS/MS; liquid chromatography-tandem mass spectrometry,

LHA; lateral hypothalamus area,

LPC; lysophosphatidylcholine,

LPS; lipopolysaccharide,

M cells; microfold cells,

2-MAG; 2-monoglyceride,

MALT; mucosa-associated lymphoid tissue,

MC-R; melanocortin receptor,

MCFA; medium-chain fatty acid,

MKK; mitogen-activated protein kinase kinase,

MMC; migrating myoelectric complex,

mRNA; messenger ribonucleic acid,

MSH; melanocortin,

Na^+; sodium ion,

NAM; negative allosteric modulator,

NAPE; N-aryl phosphatidylethanolamide,

NEFA; non-esterified fatty acid,

NF-κB; nuclear factor κB,

NF; neurofilament,

NO; nitric oxide,

NOS; nitric oxide synthase,

NPY; neuropeptide Y,

NTS; neurotensin,

OEA; oleoylethanolamide,

OG; oleoyl-glycerol,

OGTT; oral glucose tolerance test,

OLDA; N-oleoyldopamine,

OXM; oxyntomodulin,

PC; proconvertase,

Phloridzin; (1-[2-(β-D-glucopyranosyloxy)-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl]-1-propanone),

Phloretin; ((E)-1-[2,4-dihydroxy-6-[[(2R,3S,4R,6R)-3,4,6-trihydroxyoxan-2-yl]methoxy]phenyl]-3-(4-hydroxyphenyl)prop-2-en-1-one),

PI3K; phosphatidylinositol-4,5-bisphosphate 3-kinase,

PNO, pine nut oil,

Pinolenic acid; (5Z,9Z,12Z)-octadeca-5,9,12-trienoic acid,

PIP_2; phosphatidylinositol bisphosphate,

PKA; protein kinase A,

PKB; protein kinase B,

PKC; protein kinase C,

PKD; protein kinase D1,

PLC; Phospholipase C,

PLD; phospholipase D,

PepT1; proton-coupled peptide transporter,

PMD; piecemeal degranulation,

PP; pancreatic polypeptide,

po; per os,

POMC; proopiomelanocortin,

PPAR; peroxisome proliferator-activator receptor,

PSN632408; (tert-butyl 4-((3-(pyridin-4-yl)-1,2,4-oxadiazol-5-yl)methoxy)piperidine-1-carboxylate),

PVH; paraventricular hypothalamus,

PVN; paraventricular nucleus,

PVP; polyvinylpyrrolidone,

PYY; Peptide YY,

RNA; ribonucleic acid,

RYGB; Roux-en Y gastric bypass,

RT-PCR; reverse transcription polymerase chain reaction,

s.c; subcutaneous injection,

SCFA; short-chain fatty acid,

SDS; sodium dodecyl sulphate,

SGLT1; sodium-glucose transporter 1,

sIRNA; small interfering RNA,

Sitagliptin; 4-oxo-4-(3-(trifluoromethyl)-5,6-dihydro(1,2,4)triazolo(4,3-a)pyrazin-7(8H)-yl)-1-(2,4,5-trifluorophenyl)butan-2-amine,
SOM; somatostatin,

SST; somatostatin receptor,

T2DM; type II diabetes mellitus,

TA; transit-amplifying cells,

TAK1; transforming growth factor β activated kinase 1,

TAK-875; (6-((2',6'-dimethyl-4'-(3-(methylsulfonyl)propoxy)biphenyl-3-yl)methoxy)-2,3-dihydro-1-benzofuran-3-yl)acetic acid hemihydrate,
TAB1; TGF-β activated binding protein 1,

TGF-β; transforming growth factor β,

TLR; toll-like receptor,

TNF, tumour necrosis factor,

TNFR; tumour necrosis factor α receptor,

TRPV1; transient receptor potential cation channel subfamily V member 1,

TTX; tetrodotoxin,

TUG424; (3-(4-(o-Tolylethynyl)phenyl)propanoic acid),

TUG891; **(**4-[(4-Fluoro-4'-methyl[1,1'-biphenyl]-2-yl)methoxy]-benzenepropanoic acid),

UK14,304; 5-Bromo-6-(2-imidazolin-2-ylamino)quinoxaline,

UGIT; upper gastrointestinal transit,

VDCC; voltage dependent calcium channels,

VIP; vasoactive intestinal polypeptide,

VMAT; vesicular monoamine transporter,

VMH; ventromedial hypothalamus,

VPAC; vasoactive intestinal polypeptide receptor,

WT; wild-type

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CHAPTER 1

INTRODUCTION

1.1 Obesity and diabetes – a worldwide twin epidemic

Over the past decade, the prevalence of obesity (defined as having a body mass index (BMI) of >30 kg/m^2) has grown in severity in all populations. Today, obesity affects more than 650 million individuals globally, and is therefore considered a worldwide epidemic (World Health Organisation). In the UK, 2 in every 3 individuals are overweight or obese (Diabetes UK) and in the US, only a third of the population is considered to have a normal BMI (Centers of Disease Control and Prevention). The current predicament of obesity is attributed to a Western lifestyle, consisting of highly palatable and calorie-dense food and a reduction in energy expenditure (World Health Organisation). Obesity is associated with numerous co-morbidities, the most prevalent being Type II diabetes mellitus (T2DM) (Guh et al., 2009). T2DM is associated with a decline in β cell insulin secretion, hyperglycaemia, progressive β cell apoptosis and whole-body insulin resistance, resulting in a reduction in glucose uptake in the periphery (skeletal muscle, liver and adipose tissue) (Kahn et al., 2006). The term diabesity was coined to express the closely-linked relationship between obesity and T2DM (Zimmet et al., 2001). Both diseases are characterised by peripheral insulin resistance and pancreatic β cell insulin deficiency (Verma and Hussain, 2017). As early as 1997, it was revealed that for every kg of weight gained, the risk of diabetes increased by 4.5 – 9 %, highlighting a potential link between obesity and T2DM (Ford et al., 1997). Today, approximately 90 % of T2DM cases are attributed to excess weight gain (World Health Organisation), confirming weight gain as a significant contributor to an increased risk of T2DM (Al-Goblan et al., 2014).

At the start of this PhD (2014), approximately 382 million individuals were diagnosed with diabetes. It is predicted that without concerted action, the number of individuals diagnosed is expected to rise to 592 million by 2035, especially in developing countries (Guariguata et al., 2014). The exponential increase in this twin epidemic is not only a public health and economical concern, but a challenge in terms of discovering improved nonsurgical combinational therapies to curb both diseases. Diet, lifestyle and exercise modification, while initially promising, are linked to a high failure rate without long-term benefit. In clinical practise, obese/overweight patients only maintained 4.7 % weight loss with intensive behavioural therapy over 2 years (Wadden et al., 2011), demonstrating this intervention method was not an adequate treatment. There is only 1 drug available and licenced in Europe (Orlistat) and only 3

anti-obesity drugs currently available in the US (Phentermine, Orlistat, Lorcaserin) (Yanovski and Yanovski, 2014). Orlistat is a pancreatic lipase inhibitor which prevents lipid hydrolysis and absorption, but only achieves modest weight loss of 2.9 kg compared to placebo over a one year period (Rucker et al., 2007). Unfortunately, Orlistat is associated with an increase in faecal fat, also known as steatorrhea, and this severely limits patient compliance (Douglas et al., 2015). Previously licenced anti-obesity therapeutics, Sibutramine (serotonin-noradrenaline reuptake inhibitor) and Rimonabant (cannabinoid receptor 1 (CB1) antagonist) were withdrawn due to cardiovascular and psychiatric health concerns (Christensen et al., 2007; James et al., 2010). The lack of successful anti-obesity therapeutics demonstrates the critical need for new efficacious drugs in this field, without adverse effects. Currently, in the UK there are no available combinational drug formulations to treat both obesity and T2DM simultaneously.

The successful surgical treatment of obesity is limited to bariatric surgery, particularly Roux-en Y gastric bypass (RYGB). RYGB achieves efficacious and maintained long term weight loss (75 %) (Smith et al., 2008), resolution of T2DM and a reduction in mortality and morbidity (Sjöström et al., 2007). This surgical procedure involves sectioning a portion of the stomach, which forms a gastric pouch that is anastomosed to the jejunum (alimentary limb). This creates a diverted passage for ingested material to reach a more distal region of the small intestine (Meek et al., 2016). In the past, RYGB was advocated for individuals who were morbidly obese (BMI of 40kg/m^2). However, due to the steep rise in the obese population and the lack of efficacious anti-obesity therapeutics, surgery is now available to patients with a BMI >35kg/m^2, with associated severe T2DM or hypertension (Mayo Clinic, 2017). The success of RYGB has been attributed to the alteration in the milieu of gastrointestinal (GI) hormones (e.g. peptide YY(PYY) and glucagon-like peptide 1(GLP-1)) post-surgery (le Roux et al., 2006a; Saeidi et al., 2013). As a result, in the last 10-15 years, targeting the release of GI hormones, specifically PYY and GLP-1 from enteroendocrine L cells, has become a major area of interest to emulate post-RYGB GI peptide alterations and combat both obesity and T2DM (le Roux et al., 2006a; Meek et al., 2016). Proven GLP-1 mimetics (Exenatide and longer-acting Liraglutide) are market-approved anti-diabetic therapeutics that mimic the effects of GLP-1, at the glucagon-like peptide 1 receptor (GLP-1R). These drugs are well tolerated, robustly increase β cell insulin

secretion, improve glucose tolerance and show mild GI adverse effects (Chaudhury et al., 2017). GLP-1 mimetics also cause modest weight loss. In an earlier study, short-term Exenatide treatment caused 2.49 kg weight loss in a subset of obese woman, compared to 0.43 kg weight gain in the placebo-treated group (Dushay et al., 2012). More recently, Liraglutide induced 5-10 % weight loss compared to placebo (Mehta et al., 2017), indicating GLP-1 mimetics possess anti-diabetic and anti-obesity activity. PYY is a potent anorexigenic peptide. Clinical data indicated that infusion of truncated PYY (also known as $PYY_{(3-36)}$) reduced food intake in healthy volunteers, confirming the robust satiety-inducing effects of this hormone (Degen et al., 2005; le Roux et al., 2006b). These favourable anti-diabetic and anti-obesity effects of L cell-derived GLP-1 and PYY have facilitated the development of agonists that stimulate endogenous GLP-1 and PYY release. These potential agonists could emulate the effects of GLP-1 mimetics, with the added benefit of PYY-induced satiety, and therefore provide relief of both diseases (obesity and T2DM), with a single drug formulation. Lipids/lipid metabolites powerfully stimulate PYY (Beglinger and Degen, 2006) and GLP-1 release postprandially in humans (Little et al., 2005; Carr et al., 2008; Beglinger et al., 2010; Hansen et al., 2011; Mandøe et al., 2015), rodent models (Adachi et al., 2006; Xiong et al., 2013; Dailey et al., 2014; Ekberg et al., 2016; Moss et al., 2016) and primary cultured L cells (Habib et al., 2013; Moss et al., 2016). Therefore, in this thesis, the GI L cell lipid receptors, free-fatty acid receptors (FFA) 1 (FFA1, previously known as GPR40), FFA4 (GPR120) and the ethanolamide receptor, GPR119, were interrogated with novel selective agonists and antagonists.

1.2 Overview of the gastrointestinal tract

The GI tract is a group of organs from the oral cavity to the rectum (including the pharynx, oesophagus, stomach, small intestine, large intestine) and encompasses the associated glandular organs (Figure 1.1). Together, they form the largest endocrine organ of the human body, that at its luminal interface consists of a single layer of epithelial cells (Reed and Wickham, 2009). This epithelial layer is in direct contact with the external environment and exhibits a large surface area of 100 – 400 m^2 (includes the stomach, small intestine and colon) (Ferraris et al., 1989; MacDonald and Monteleone, 2005; Artis, 2008). Each GI area has a crucial role to play in energy

homeostasis, digestion, nutrient (fatty acids, amino acids and monosaccharides) and electrolyte absorption and secretion, monitoring the energy status of the body, activating and modulating the appropriate physiological responses, protecting the body from bacteria and carcinogens and eliminating undigested material (Furness et al., 2013). Ingested material is temporarily stored in the stomach before it is released into the small intestine, the primary region of nutrient digestion and absorption. Here, lipids, proteins and carbohydrates undergo enzymatic digestion, providing fatty acids, amino acids and monosaccharides, respectively (Hall, 2011). Absorption of these digested nutrients from the intestinal lumen, stimulates the release of a myriad of peptides that are key regulators of satiety, GI motility, gastric emptying, enteroendocrine and exocrine secretions and overall control of body weight (Perry and Wang, 2012). Finally, the remaining undigested luminal material reaches the large intestine, a GI region mainly responsible for reclaiming luminal fluid and electrolytes. The undigested material is temporarily stored in the sigmoid colon and rectum, prior to excretion (Barrett and Raybould, 2010).

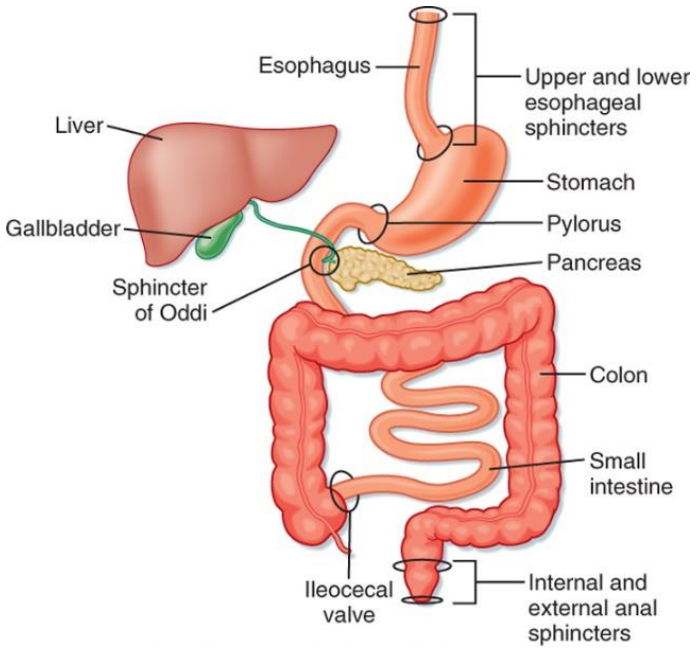


Figure 1.1: The structure of the GI tract. The oral cavity to the anal sphincter, including the associated glandular organs (the liver, pancreas and gallbladder). Image from Barrett and Raybould (2010).

1.3 The small intestine

The small intestine constitutes the area from the pyloric sphincter to the ileocaecal junction. In humans, the small intestine is approximately 5 m in length and is divided into three regions, the duodenum, the jejunum and the ileum (Mescher, 2010). As chyme arrives in the duodenum, its acidity is neutralised by bicarbonate ions (HCO_3^-) secreted mainly by the pancreas and gallbladder, via the pancreatic duct and common bile duct, respectively (Reed and Wickham, 2009; Barrett and Raybould, 2010). Additionally, duodenal submucosal Brunner glands contribute to the neutralisation of chyme by secreting an alkaline fluid (pH 8.1 - 9.3). This protective mechanism shields the mucous membrane and ensures chyme is at an optimum pH for pancreatic enzyme activity (Mescher, 2010). The duodenum and jejunum are responsible for achieving complete digestion of lipids, carbohydrates and proteins to allow efficient absorption of their digested nutrients (Hall, 2011). The robust nutrient absorption in these regions is attributed to the increased surface area provided by mucosal villi (0.5 to 1.5 mm long) that project into lumen and secondly, microvilli (1 µm tall and 0.1 µm in diameter) that protrude from the apical cytoplasm of absorptive enterocytes

(MacDonald and Monteleone, 2005; Mescher, 2010). Each enterocyte contains 3000 microvilli and therefore 1 mm^2 of intestinal mucosa is home to 200 million microvilli, vastly increasing the area of contact with ingested nutrients, allowing efficient absorption (Mescher, 2010).

1.3.1 Digestion of lipids

As triglycerides enter the small intestine, they are broken down into smaller lipid micelles (0.5 µm in diameter) (Carey et al., 1983) and phospholipids by mechanical disruption, caused by the frequent contractions of the stomach and small intestine (Hall, 2011). The newly arrived bile, secreted from the gallbladder, contains bile salts that prevent reaggregation of the smaller lipids, a process known as emulsification (Iqbal et al., 2009) (Figure 1.2). Due to the amphipathic nature of phospholipids and bile salts, the lipolytic enzyme, pancreatic lipase is repelled (Chapus et al., 1975). To combat this, a second pancreatic enzyme, colipase, binds the emulsified lipid droplet and provides a binding site for pancreatic lipase (Erlanson-Albertsson, 1992; Lowe, 1997). This lipase digests triglycerides, yielding 2-monoglyceride (2-MAG) and two FFAs (Mattson and Beck, 1955; Mattson and Volpenhein, 1964; Clément, 1976). These lipid metabolites, phospholipids and bile salts aggregate to form micelles. Micelles continuously disaggregate and re-form, which enhances their absorption into the enterocytes (Clément, 1976; Mu and Høy, 2004). The lipid nutrients are detected and sensed by GI lipid-sensing receptors i.e. FFA1, FFA4 and GPR119 (Ekberg et al., 2016). Within the enterocytes, the lipid metabolites, 2-MAG and FFAs re-esterify forming chylomicrons (Mattson and Volpenhein, 1964). Chylomicrons are exocytosed into the interstitial fluid and transported into the lymphatic system.

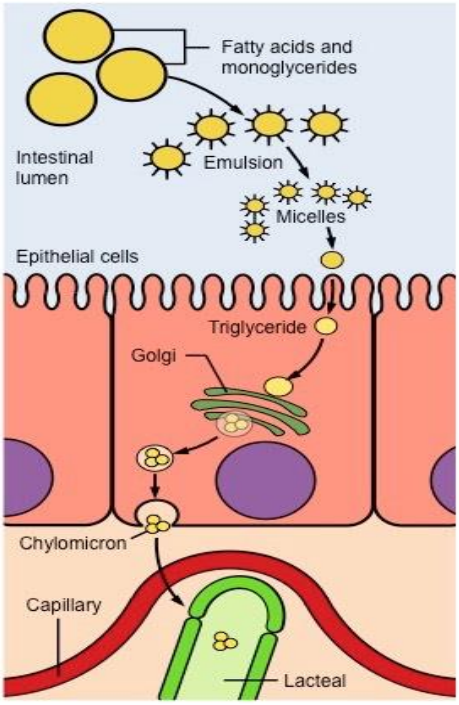


Figure 1.2: Digestion of triglycerides in the small intestine. Large lipid droplets are broken down by mechanical disruption into smaller lipids. Bile salts released from the gallbladder create an emulsification mixture that prevents re-aggregation of the small lipids. Pancreatic lipase digests triglycerides into 2-MAG and two FFAs. These lipid metabolites along with phospholipids and bile form micelles. Finally, micelles are absorbed into intestinal enterocytes. Image from http://ib.bioninja.com.au/standard-level/topic-6-human-physiology/61-digestion-and-absorption/lipid-absorption.html.

1.3.2 Digestion of carbohydrates

Ingested carbohydrates consist of monosaccharides, disaccharides and polysaccharides. Monosaccharides are absorbable and do not require further digestion. In contrast, disaccharides (sucrose) and polysaccharides (e.g. starch) require enzymatically degradation before absorption can occur. The pancreatic enzyme, α-amylase digests disaccharides and polysaccharides into shorter chains of glucose e.g. maltose, lactose and sucrose (Figure 1.3) (Dahlqvist and Borgstrom, 1961). The shorter chains of glucose undergo further digestion by disaccharidases

(sucrase, maltase and lactase), found embedded in the intestinal microvilli brush border to yield monosaccharides (Holmes, 1971). Sucrase digests sucrose into glucose and fructose. Maltase yields two glucose molecules from maltose, and lactase splits the bond between galactose and glucose (Holmes, 1971; Dahlquist and Semenza, 1985; Levin, 1994). Glucose (as well as galactose) is actively transported across enterocytes predominantly via the apical-located sodium-glucose transporter 1 (SGLT1) (Crane, 1962; Wright et al., 2011; Gorboulev et al., 2012), whereas fructose is transported across the epithelia via the luminal facilitative transporter, GLUT5 (Davidson et al., 1992; Miyamoto et al., 1993). As the glucose concentration increases, the facilitative transporter, GLUT2 is trafficked to the luminal membrane to facilitate glucose absorption in rodents (Kellett and Helliwell, 2000; Tobin et al., 2008; Gorboulev et al., 2012; Mace et al., 2012). Absorbed glucose, galactose and fructose exit the enterocytes and enter the circulation via serosal GLUT2 (Thorens et al., 1990; Wright et al., 2011).

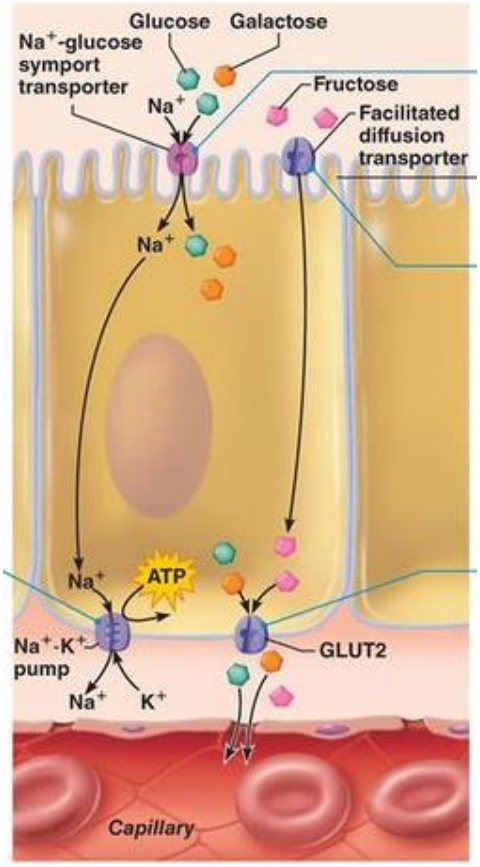


Figure 1.3: Digestion of carbohydrates in the small intestine. Polysaccharides (starch, glycogen) are digested by pancreatic α-amylase in the intestine into disaccharides (maltose, sucrose and lactose). Disaccharides are further digested by disaccharidases (maltase, sucrase, lactase) located in the microvilli brush border into monosaccharides. Image from https://www.pinterest.co.uk/pin/751678994025683223/.

1.3.3 Digestion of proteins

In the duodenum and jejunum, ingested proteins and polypeptides are digested into oligopeptides and amino acids by pancreatic proteolytic enzymes (trypsin, chymotrypsin, elastase and carboxypolypeptidase), excreted via the hepatopancreatic sphincter. Trypsin, chymotrypsin and elastin are serine proteases. Trypsin cleaves peptide bonds adjacent to lysine or arginine, whereas chymotrypsin cleaves peptide bonds next to hydrophobic amino acids to yield oligopeptides. Furthermore, elastase cleaves elastin and peptide bonds next to alanine, glycine and serine, and carboxypolypeptidase digests the carboxyl ends of peptides, releasing free amino acids **(**Figure 1.4) (Whitcomb and Lowe, 2007; Goodman, 2010). Final protein hydrolyses

occurs at the enterocyte brush border membrane by aminopeptidase (hydrolysing the amino-terminus) and dipeptidases (cleaves dipeptides into free amino acids) (Tobey et al., 1985; Goodman, 2010). The free amino acids are transported across enterocytes into the circulation via several electrogenic amino-acid transporters, while approximately 8000 distinct di- and tripeptides are transported via the high capacity/low affinity proton-coupled peptide transporter, PepT1 (Liang et al., 1995; Fei et al., 2000; Spanier, 2014).

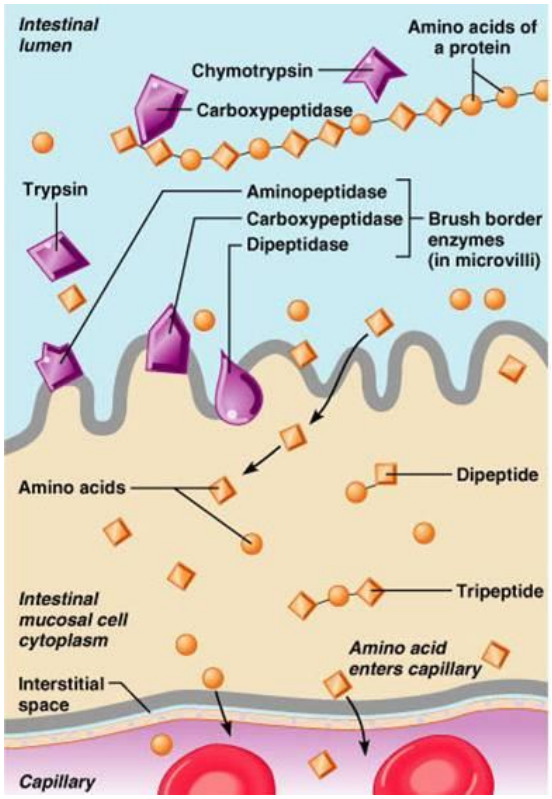


Figure 1.4: Protein digestion in the small intestine. Pancreatic proteolytic enzymes (trypsin, chymotrypsin, carboxypeptidase) and microvilli brush border enzymes (aminopeptidase, carboxypeptidase) digest proteins into di- and tripeptides and free amino acids. The digested proteins are transported across the enterocyte plasma membrane and into the circulation. Image from http://www.austincc.edu/apreview/PhysText/Digestive.html.

1.4 The large intestine

The large intestine of humans (from the ileocaecal junction to the rectum) spans a total length of approximately 1.5 m, and is divided into the caecum, ascending colon, hepatic flexure, transverse colon, splenic flexure, descending colon, sigmoid colon and the rectum (Geissler and Powers, 2017). The colon has multiple invaginations with flat intercrypt tables (Figure 1.5) and is home to approximately 1200 different bacterial species, with 160 microbiota unique to each host (Qin et al., 2010; Schroeder and Bäckhed, 2016). Collectively, the microbial genome is greater than the human genome, encoding at least 100 times more genes (Ley et al., 2006; Qin et al., 2010). In the intestine, the quantity and diversity of the microbiota increases along the length of the GI tract, from the small intestine (duodenum, $10^1 – 10^3$; jejunum/ileum, $10^4 – 10^7$ per ml of luminal contents) to the large intestine ($10^{11} – 10^{13}$ per ml of luminal contents) (Whitman et al., 1998; Eckburg, 2005; O 'hara and Shanahan, 2006; Artis, 2008; Donaldson et al., 2016; Sender et al., 2016). The two major phyla systems in the distal gut that constitute 90 % of phyla in this region, are Firmicutes and Bacteroidetes. The quantitative ratio between these two phyla vary between hosts (Eckburg, 2005). The complexity increases as differences in mucosal surface-adherent and lumen microbiota populations have been observed, which also vary in aerobic and anaerobic ratios (Eckburg, 2005). The colonic microbiota critically degrade complex indigestible carbohydrates, yielding short-chain fatty acids (SCFA) such as acetate, propionate and butyrate (Marchesi et al., 2016). The SCFAs are predominantly absorbed via passive diffusion, however some require active transport via various SCFA transporters. SCFA are also sensed by FFA receptor 2 (FFA2) and 3 (FFA3) to regulate lipid and glucose metabolism (Brown et al., 2003; Ulven, 2012). Oxidation of absorbed SCFAs, particularly butyrate provides 60-70 % of the energy supply of colonic enterocytes. Thus, SCFAs are a fundamental energy source in the colon (den Besten et al., 2013).

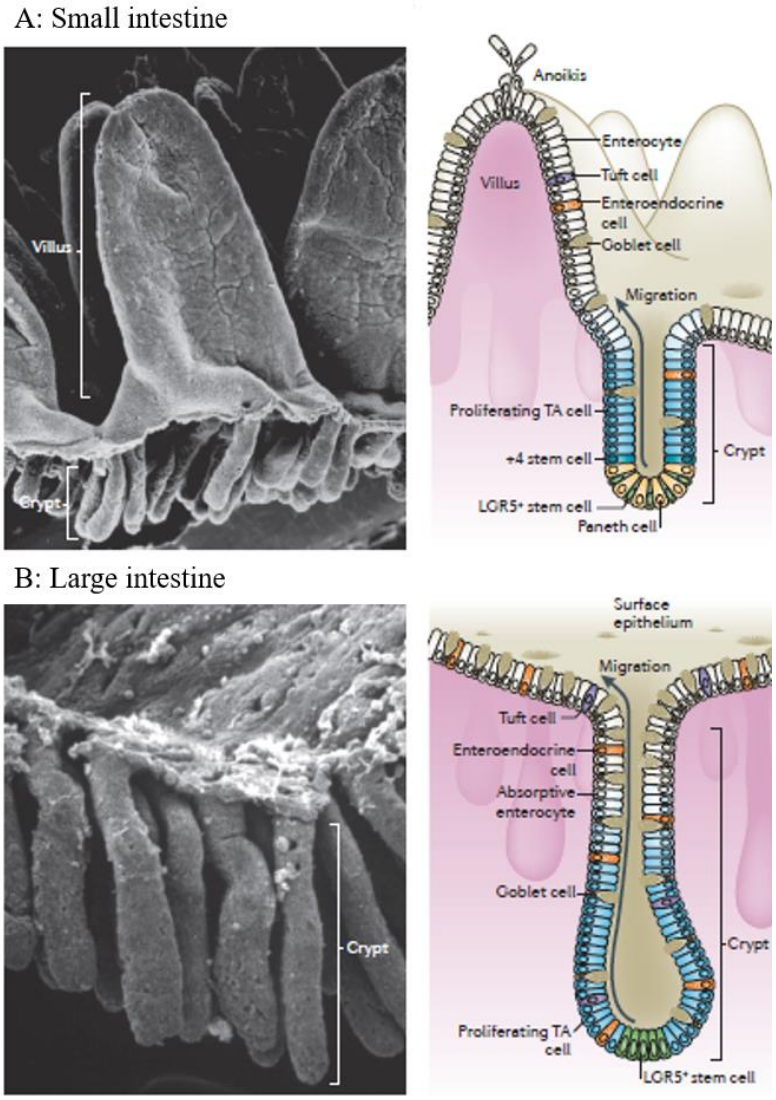


Figure 1.5: The structural organisation of the small intestine (A) and colon (B) in mouse, shown in the scanning electron micrograph panel on left and in the schematic on the right. The small intestine contains specialised villi and microvilli that vastly enhance the surface area of the epithelium and are specifically required for efficient absorption of digested nutrients. Conversely, the colon consists of flat laminar endings (intercrypt tables) and lacks intestinal villi. Image from Barker (2013).

1.5 Structural organisation of the intestine

The structural organisation of the intestine is composed of the mucosa, submucosa, submucosal plexus, the muscularis externa, myenteric plexus and the serosa, which forms part of the mesentery (Figure 1.6) (Mescher, 2010).

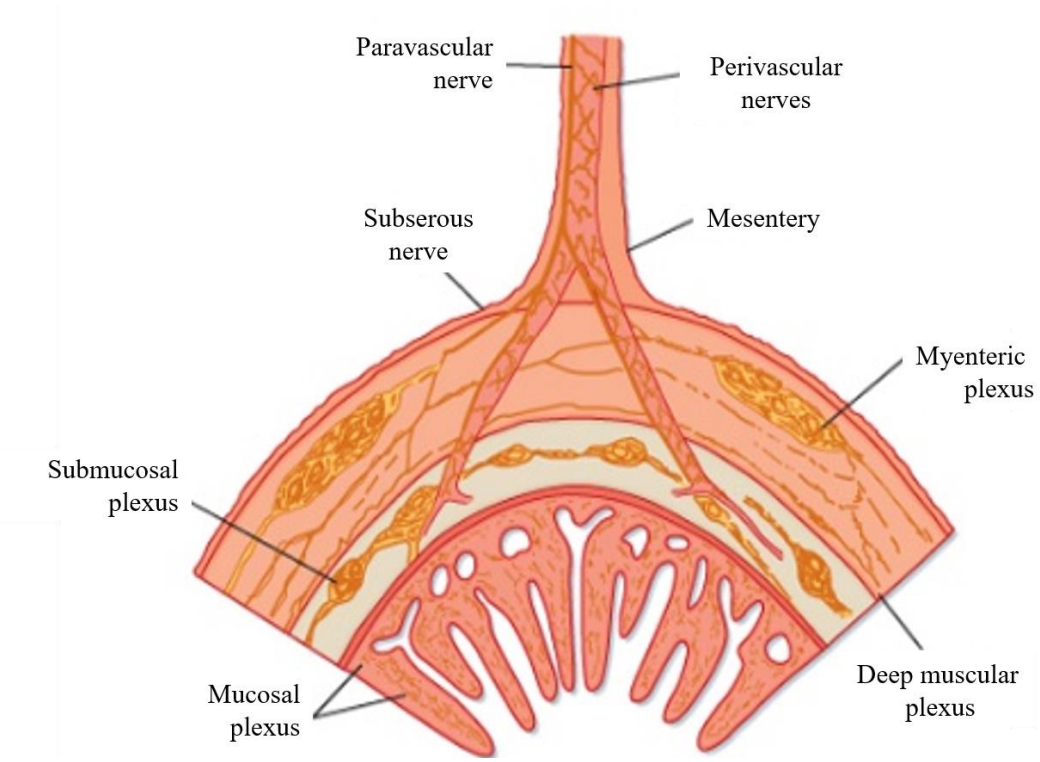


Figure 1.6: Transverse section illustrating the anatomy of the small intestine. Structural layers of the GI tract include the mucosa (epithelium, lamina propria, muscularis mucosa), submucosa with the associated submucosal plexus, muscularis externa (constituting circular and longitudinal smooth muscle) and a second neuronal plexus, the myenteric plexus lies between the two smooth muscle layers. The serosa is the most outer layer that forms part of the mesentery. Image from Barrett and Raybould (2010).

1.5.1 *The intestinal mucosa*

The mucosa is the innermost structural layer of the intestine and is highly adaptable to the ever-changing macronutrient environment in the GI lumen. It provides mucosal

immunity against noxious substances, aids digestion and absorption of ingested material, maintains mucosal integrity via tight junctions and regulates electrolyte and fluid transport, which critically prevents hypo- or hypersecretion states (Hall, 2011). The mucosa is segregated from the GI lumen by a single layer of simple columnar epithelial cells, surrounded by the lamina propria and muscularis mucosa (Mescher, 2010). Here, the different cell populations along the length of the GI tract epithelium are organised to meet the functional requirements of specific regions and are linked by highly selective intercellular tight junctions. These tight junctions are composed of occludins (Furuse et al., 1993), claudins (Furuse et al., 1998), junctional adhesion molecules (Martìn-Padura et al., 1998) and tricellulin (Ikenouchi et al., 2005), which together critically regulate cell permeability, paracellular transport of fluid and electrolytes and prevent bacterial translocation. Importantly, these tight junctions segregate the luminal and serosal membrane and compartment (Nusrat et al., 2000). In this thesis, the segregation of luminal (also known as apical (ap)) and serosal (also known as basolateral (bl)) epithelial surfaces imposed by the tight junctions, allowed interrogation of various side-specific mucosal receptors with selective agonists.

In humans, the GI epithelium undergoes a daily cell death of 10^{11} epithelial cells (Potten, 1995), and therefore it is pertinent that the epithelium adapts and self-renews to maintain optimum barrier function. The epithelium is steadily replaced during adulthood with newly regenerated cells, originating from 4-6 adult stem cells per crypt. These stem cells are anchored at the base of discrete proliferative invaginations (bottle-shaped), known as the crypts of Lieberkühn (Marshman et al., 2002). Initial studies labelled proliferating cells with [^{3}H]thymidine to show cell migration and intestinal epithelium renewal. This revealed stem cells lie at the base of each crypt (Cheng and Leblond, 1974). These pluripotent stem cells give rise to a transient population of highly proliferative progenitor cells, known as transit-amplifying cells (TA). These TA cells undergo 2-3 cell divisions and terminally commit to one of four linages, while migrating towards the lumen (with the exception of Paneth cells) (Cheng and Leblond, 1974) (Figure 1.7). The four lineages include the absorptive cell, enterocytes (80 %) (Karam, 1999; de Santa Barbara et al., 2003) or one of the three secretory lineages, nutrient sensing enteroendocrine cells (EECs) (<1 %) (Buffa et al., 1978), mucus-secreting goblet cells (5 % in small intestine (de Santa Barbara et al., 2003) and 16 % in the colon (Karam, 1999)) and immune Paneth cells. Cup cells

(6 %) (Madara, 1982), tuft cells (0.4 %) (Gerbe et al., 2012), Peyer-patch cells (46 %) (Van Kruiningen et al., 2002) and microfold cells (M cells; 5-10 %) (Ohno, 2016) have also been identified, but their functions are still poorly defined. Once the terminal differentiated cell types reach the top of small intestinal villi or the colonic intercrypt table, they are sloughed off into the lumen, a process occurring every 3-5 days in mammals (Potten, 1995). Conversely, Paneth cells migrate towards the base of the crypt (Bjerknes and Cheng, 2005). Here, they secrete eosinophilic secretory granules containing lysozymes, phospholipase A_2 and defensins (Mescher, 2010). Initial [^{3}H]thymidine labelling studies in mouse established Paneth cell renewal occurs every 18 - 22 days (Cheng et al., 1969). Later, Ireland *et al.* (2005) indicated Paneth cell turnover occurs every 57 days in mice, three-times long than initial findings. Recently, Bohórquez *et al.* (2015), revealed a similar turnover rate for a population of long-lived mouse EECs. These cells were more resilient and could reside in the epithelium for up to 60 days (Bohórquez et al., 2015). Understanding the longer lifespan of these EECs in the epithelial lining while other EECs/enterocytes are replaced, is yet to be elucidated.

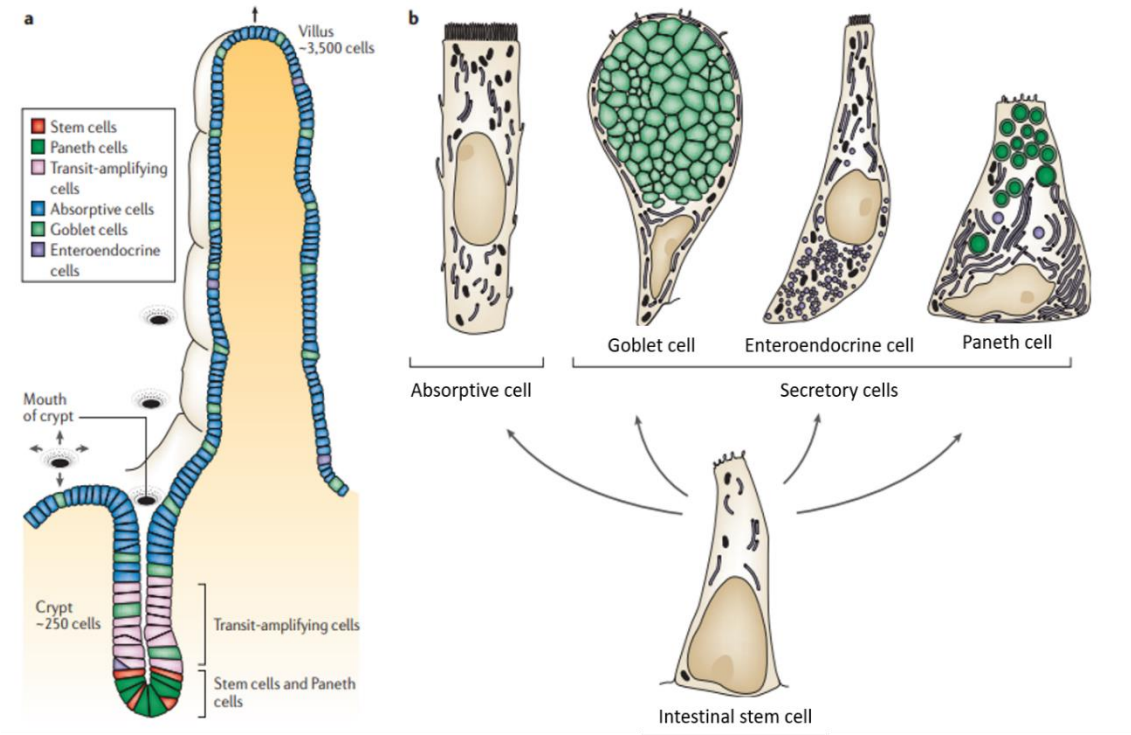


Figure 1.7: The distribution of various epithelial cell types along the small intestine. Stem cells anchored to the base of crypts of Lieberkühn divide and migrate to produce proliferative progenitors also known as TA cells (partially differentiated). These TA cells undergo terminal differentiation to produce 1 of 4 lineages: enterocytes, goblet cells, EECs or Paneth cells. Image from Crosnier *et al.* (2006).

The lamina propria segregates the mucosal epithelium from the submucosa and penetrates each intestinal villus. This layer is primarily composed of loose connective tissue, fibroblasts, blood and lymphatic vessels, nerve fibres, lymphoid nodules, dendritic cells, antigen presenting cells, immunoglobulin A-secreting plasma cells, macrophages and lymphocytes (B cells and T cells) (Reed and Wickham, 2009; Mescher, 2010). Surrounding the lamina propria is the outermost layer of the mucosa known as the muscularis mucosa. This layer is composed of elastin fibres and 3-10 smooth muscle cells (Boudry et al., 2004). These smooth muscle cells may extend into the intestinal villi and their rhythmic contraction is responsible for the movement of the villi (Mescher, 2010).

In the ileum and colonic lamina propria (and submucosa), aggregates of lymphoid follicles (10-200) form Peyer patches, an important component of mucosa-associated lymphoid tissue (MALT) (Mescher, 2010). Together, Peyer patches, mesenteric lymph nodes and the appendix are collectively known as gut-associated lymphoid tissue (GALT) (Guy-Grand et al., 1974). The GALT is responsible for 50 % of the body's immune response, providing mucosal immunity against resident bacteria and preventing penetration of pathogens from the external environment (Fukatsu and Kudsk, 2011). M cells play an important role in GI mucosal immunity. Simple follicle-associated epithelium (FAE), containing M cells lie above Peyer patches. M cells contain specialised apical folds and a basolateral intraepithelial pocket, open to underlying aggregates of lymphoid tissue via a porous basement membrane (Neutra and Kraehenbuhl, 1992). These cells sample luminal antigenic ingested material and pathogens. Antigens that bind to M cells are translocated into the intraepithelial pocket and are subsequently presented to the underlying immune cells. This initiates an adaptive immune response to neutralise the presented antigens (Neutra and Kraehenbuhl, 1992; Neutra et al., 1996).

1.5.2 Submucosal plexus and deep muscular plexus

The submucosa is a thin layer of loose connective tissue that supports the mucosa and consists of blood vessels, lymph vessels and submucosal glands (i.e. duodenal Brunner glands) (Barrett and Raybould, 2010). The blood vessels extend from this region to the intestinal villi forming a capillary network just beneath the epithelium. Venules branch off from this network to re-join the submucosal veins. The lymphatic vessels also known as lacteals begin in the intestinal villi, extend to the lamina propria where they form a plexus, and eventually are directed to lymphoid nodules in the submucosa (Mescher, 2010). Surrounding the submucosa is an intrinsic neuronal network known as the submucosal plexus (also known as Meissner plexus) (Furness and Costa, 1987). In humans, this plexus is interconnected with a second submucosal plexus also known as Henle's plexus, located closest to the circular muscle (Hoyle and Burnstock, 1989). Encircling the submucosa, is a smooth muscle layer, known as the muscularis externa. This muscle layer is made up of two layers of smooth muscle, the inner layer consists of circular muscle fibres and the outer layer is composed of longitudinal muscle fibres.

In humans, the circular muscle layer is further divided into the inner (more densely packed) and outer circular muscle layers (Furness and Costa, 1987). Interspersed in the muscularis externa between the circular and longitudinal muscle cells are interstitial cells of Cajal (ICC), also known as the pacemaker cells of the muscularis externa (Sanders et al., 2006). ICCs are responsible for generation, modulation and propagation of slow wave potentials that regulate GI motility (Farrugia, 2008). Between the circular and longitudinal muscle layers is a second intrinsic network known as the myenteric plexus (also known as Auerbach plexus). Together, the myenteric plexus and the submucosal plexus form the intrinsic nervous system of the GI tract, also known as the enteric nervous system (ENS) (Furness and Costa, 1987; Furness et al., 2014).

1.5.3 Serosa

Surrounding the muscularis externa lies the serosa, a thin layer of squamous mesothelial cells and loose connective tissue. This layer forms part of the mesentery, which surrounds the intestine providing protection and support (Barrett and Raybould, 2010).

1.6 The enteric nervous system

The ENS of the GI tract forms the third division of the autonomic nervous system (ANS) and is composed of an interconnected intrinsic circuit of ganglionated neurons (400-600 million in humans) and glial cells (Furness and Costa, 1987; Furness, 2006), which are able to function independently of the central nervous system (CNS) (Bayliss and Starling, 1899). While the ENS is often referred to as an autonomous nervous system, local enteric reflexes integrate with autonomic reflexes from the CNS, to regulate gastric acid secretion, gastric emptying, GI motility, appetite and satiety (also known as the gut-brain-axis), local blood flow, fluid and electrolyte secretion, expulsion of noxious substances, enteroendocrine secretions, immune responses (Furness, 2012), mucosal integrity (Toumi et al., 2003) and defaecation (Furness and Costa, 1987; Furness, 2006).

The ENS neurons and glia are composed of two main ganglionated plexuses (Brehmer et al., 2010), the submucosal plexus (also known as Meissner plexus) located adjacent to the mucosal layer, and the myenteric plexus (Auerbach plexus) situated between the longitudinal and circular smooth muscle layers of the muscularis externa (Furness and Costa, 1987). The submucosal plexus can be subdivided into two intensively interconnected plexuses. First, the inner plexus which is nearest to the muscularis mucosa (Meissner plexus) and a second outer plexus (Schabadasch plexus, also known as Henle's plexus) observed in larger mammals, found adjacent to the circular smooth muscle of the muscularis externa (Hoyle and Burnstock, 1989). Notably, the submucosal plexus has been identified in the small and large intestine and is rarely seen in the stomach and oesophagus. In contrast, the myenteric plexus is found throughout the GI tract, from the oesophagus to the rectum sphincter (Furness, 2012). There are three functional classes of neurons that have been identified in the ENS: intrinsic primary afferent neurons (IPANs), motor neurons and interneurons (Furness, 2000; Furness, 2008; Qu et al., 2008; Furness et al., 2014) (Table 1.1). Many of the initial ENS studies were carried out in guinea pig, attributed to the accessibility of large ENS plexuses, of which many of the enteric neurons types (approximately 14) have been identified (Furness and Costa, 1987; Furness, 2000). However, over the years the mouse has become the species of choice as genetic manipulation and the generation of models of disease are easily accessible (Gershon, 1999). Characterisation of the mouse ENS revealed that the neuronal projections, morphology, neurochemistry and electrophysiology closely resembled the human and guinea-pig ENS, therefore providing a comparative model (Sang and Young, 1996; Sang et al., 1997; Sang and Young, 1998; Furness et al., 2004; Nurgali et al., 2004; Qu et al., 2008; Mongardi Fantaguzzi et al., 2009).

Mouse small intestine		
Neuronal type	**Chemical code**	**Proportion**
IPANs	ACh/NF/CGRP/calbindin ± calretinin	26 %
Inhibitory circular muscle motor neuron	NOS/VIP ± NPY	23 %
Inhibitory longitudinal muscle motor neuron	NOS/VIP	3 %
Excitatory circular muscle motor neuron	ACh/neurokinins ± calretinin	21 %
Excitatory longitudinal motor neuron	ACh/calretinin ± neurokinins	13 %
Descending interneuron	ACh/NOS	3 %
Descending interneuron	ACh/5-HT	1 %
Descending interneuron	ACh/SOM/calretinin	4 %
Ascending interneuron	ACh/neurokinins ± calretinin	4 % (estimated)

Table 1.1 Different ENS neuronal populations in the mouse small intestine. Table 1.1 shows the various types of ENS neurons in the mouse small intestine, including the chemical code and the proportion of each neuronal type. Table from Qu *et al.* (2008).

1.6.1 Intrinsic primary afferent neurons

In the mouse, Dogiel type II multi-axonal neurons (also known as AH neurons) closely resemble the morphology, projections, chemistry coding (Furness et al., 2004) and electrophysiological properties (Nurgali et al., 2004) of the guinea pig Dogiel type II IPANs. As a result, mouse Dogiel type II neurons are most likely IPANs (Furness et al., 2004; Nurgali et al., 2004). Mouse IPAN cell bodies are oval-shaped and project short and long axons. IPANs constitute 20.6 % of the total myenteric neuronal population in mouse colon (Nurgali et al., 2004) and 26 % in the mouse small intestine

(Qu et al., 2008). The population of IPANs appears to be negatively correlated to species size, with larger mammals possessing fewer IPANs. Indeed, in humans the IPAN population comprises 10 % of the total neuronal population (Weidmann et al., 2007), approximately half the quantity of IPANs observed in the mouse. Colonic (Furness et al., 2004) and small intestinal (Qu et al., 2008) IPANs project to the myenteric ganglia and the mucosa (Furness et al., 2004; Nurgali et al., 2004). Similarly, human intestinal IPANs project to the mucosa, which was illustrated utilising retrograde transported dye (Dil) (Hens et al., 2001). In an earlier study, stimulation of guinea pig small intestine mucosal nerve fibres provided action potentials which were recorded from Dogiel type II neurons, confirming IPAN axons extend to the mucosa (Bertrand et al., 1998). In contrast, there appear to be no IPANs or IPAN-like neurons in the mouse submucosal layer of the ileum (Mongardi Fantaguzzi et al., 2009) or distal colon (Foong et al., 2014). Small intestinal IPANs possess the chemical coding of acetylcholine (ACh)/calbindin/ calcitonin gene-related peptide (CGRP)/neurofilament (NF) 145 kDa ± calretinin (Qu et al., 2008) and similarly, colonic IPANs are immunoreactive for CGRP/calbindin/ACh/NF±calretinin (Furness et al., 2004). In the guinea pig, IPANs are responsible for sensing mechanical distortion of the mucosa, altering enteroendocrine secretions, modulating GI motility, regulating local blood flow and fluid, as well as adjusting electrolyte secretion (Furness, 2012; Furness et al., 2014). Thus, as murine IPANs are closely related to guinea pig and human IPANs, their functional significance may be similar among these species.

1.6.2 Motor neurons

Motor neurons are Dogiel type I uni-axonal, S-type and receive fast excitatory synaptic potentials (Nurgali et al., 2004). These neurons possess broad lamellar dendritic structures, which are less extensive compared to human Dogiel type I neurons (Furness and Costa, 1987). In mammals, motor neurons are divided into excitatory (containing ACh and neurokinins) and inhibitory neurons (containing nitric oxide synthase (NOS) and vasoactive intestinal polypeptide (VIP)) (Furness, 2006). In the mouse intestine, the ratio of excitatory motor neurons to inhibitory motor neurons in circular smooth muscle is 21 %: 23 % (Qu et al., 2008) (guinea pig 12 % :16 % (Furness, 2000)) and

in longitudinal smooth muscle, 13 %: 3 % (Qu et al., 2008) (guinea pig 25 %: 2 % (Furness, 2000)). The chemical coding of the excitatory motor neurons are ACh/neurokinins ± calretinin (circular muscle) and ACh/calretinin ± neurokinins (longitudinal muscle). Conversely, the chemical coding of inhibitory motor neurons is NOS/VIP ± neuropeptide Y (NPY) (circular muscle) (Sang et al., 1997; Qu et al., 2008) and NOS/VIP (longitudinal muscle) (Qu et al., 2008). This inhibitory NOS/VIP immunoreactivity is similarly observed in humans (Timmermans et al., 1994; Porter et al., 1997). In mammals, NOS and choline acetyltransferase (ChAT) are the rate-limiting enzymes in the pathways that synthesise the inhibitory neurotransmitters, nitric oxide (NO) and the excitatory neurotransmitter, ACh, respectively (Furness, 2006). Initial mouse studies demonstrated that NOS-containing neurons projected anally (Sang et al., 1997), and ACh-containing neurons projected orally (Sang and Young, 1998), revealing inhibitory and excitatory neurons, respectively. Notably, in small mammals the predominant motor neuron innervation originates from the myenteric plexus, whereas large mammals have a small proportion of motor innervation that arises from the submucosal plexus as well (Furness, 2000).

1.6.3 Secretomotor neurons

In the mouse, there are two functional classes of secretomotor neurons (S-type) (Foong et al., 2014), namely cholinergic and non-cholinergic (Furness, 2000; Mongardi Fantaguzzi et al., 2009; Foong et al., 2014; Furness et al., 2014). The proportion of cholinergic neurons (contains 2 axons) increases distally in the colon, whereas non-cholinergic VIP-containing neurons (contains 1 axon) are vastly distributed throughout the colon (Foong et al., 2014). These two neuronal populations project their axons to the intestinal mucosa (Foong et al., 2014), and therefore secretomotor neurons are intact in the mucosal preparations utilised in this thesis. Here, secretomotor neurons importantly regulate electrolyte secretion to ensure that luminal fluid secretion is matched by absorption (Furness, 2000; Furness, 2012; Furness et al., 2014). The primary transmitter localised within cholinergic neurons is ACh, a known stimulant of electrolyte secretion and vasodilation (Foong et al., 2014). In the mouse ileum submucosal plexus, VIP non-cholinergic secretomotor neurons constitute 51 % of the total neuronal population (Mongardi Fantaguzzi et al., 2009), whereas in the

distal colon these neurons make up 80 % of the population. (Foong et al., 2014). VIP causes $G_{\alpha s}$-coupled electrolyte and fluid secretion (via vasoactive intestinal polypeptide receptors (VPAC)) and induces vasodilation of submucosal arterioles. Hyper-secretion induced by VIP causes life-threating diarrhoea and therefore careful regulation of fluid secretion is vital to survive and thrive (Foong et al., 2014).

In the mouse ileum, investigations into the classes of secretomotor submucosal neurons revealed three populations: cholinergic secretomotor (30 %: code ACh/CGRP/somatostatin (SOM)/calretinin, also small population with NPY), non-cholinergic secretomotor (20 %: code VIP/NPY/tyrosine hydroxylase(TH)) and non-cholinergic vasodilator (30 % VIP/NPY/calretinin). While only approximately 50 % of cholinergic neurons were CGRP immunoreactive in the ileum (Mongardi Fantaguzzi et al., 2009), all of the cholinergic neurons in the colon were CGRP immunoreactive (Foong et al., 2014). This suggests two populations of cholinergic neurons exists in the ileum (Mongardi Fantaguzzi et al., 2009) and only one population exists in the colon (Foong et al., 2014).

1.6.4 Interneurons

In the ENS, uni-directional interneurons (S-type) transmit GI signals between motor neurons, providing a passage for each signal to travel over the length of the GI tract. In the mouse, there appears to be four classes of interneurons, one ascending and three descending. Moreover, the chemical coding of these interneurons is strikingly similar to the guinea pig interneuron coding (Furness, 2000; Qu et al., 2008). Together, the interneurons make up approximately 10 % of the ENS neurons. The ascending interneurons (estimated 4 %) are immunoreactive for ACh/neurokinins ± calretinin. In contrast, three populations of descending interneurons exist: ACh/NOS (3 %), ACh/serotonin (5-HT) (1 %) (Sang and Young, 1996; Sang et al., 1997; Sang and Young, 1998; Qu et al., 2008) and ACh/SOM/calretinin (4 % (Van Op Den Bosch et al., 2008)) (Sang and Young, 1996; Qu et al., 2008). Interneurons are involved in local propulsion reflexes, regulate secretomotor and motility reflexes and modulate propagation of the migrating motor complex (MMC, also known as the migrating myoelectric complex). In mammals, the MMC describes the rostro-claudal GI contractions during the interdigestive state that passes through the lumen, from

stomach to ileum every 80-120 min. The MMC facilitates the movement of undigested material to the descending regions of the GI tract (Furness, 2006; Furness, 2012; Furness et al., 2014). This clears the intestinal lumen and prevents bacterial overgrowth.

1.6.5 Vagal innervation of the GI tract and intestinofugal GI neurons

The human vagus contains 40 000–50 000 axons, 90 % of which are afferent processes (Furness et al., 2014). Together, afferent axons and efferent axons form a bidirectional signalling pathway between the gut and the brain. The pre-ganglionic vagal innervation creates a rostro-caudal gradient in the GI tract, with the largest vagal innervation found in the upper GI tract (oesophagus, stomach, pancreas and gallbladder) and sparse innervation in the small and large intestine (Berthoud et al., 1991). Parasympathetic vagal afferents are low threshold tension detectors, which modulate normal GI function (Andrews, 1986). Their main functions include, relaxation of the oesophagus sphincter, propagation and propulsion of ingested material within the oesophagus, increasing gastric capacity and facilitating antral stomach contractions to relax the pylorus, increasing gastric acid secretion, contracting the gallbladder and also stimulating pancreatic enzyme secretion (Furness and Costa, 1987; Stakenborg et al., 2013; Furness et al., 2014).

Intestinofugal neurons are mechanosensory and form a distinct set of myenteric ganglia that modulate normal GI function. The cell bodies of these neurons are present in the GI wall and their axons are arranged in parallel to circular muscle fibres (Figure 1.8). Intestinofugal neurons are activated by stretch/distension of circular muscle or the presence of fatty acids in the distal intestinal region. Activation of intestinofugal neurons initiates entero-enteric reflexes that modulate proximal intestinal function (Szurszewski et al., 2002). Activated intestinofugal neurons project their afferent axons to sympathetic prevertebral ganglia and synapse with post-ganglionic sympathetic fibres. The sympathetic fibres project back to the GI tract via entero-enteric pathways to inhibit fluid secretion and intestinal motility, in the proximal intestine (Furness, 2006). Taken together, the entero-enteric reflex is an essential stimulus of the intestinal brakes and ensures maximum nutrient absorption occurs.

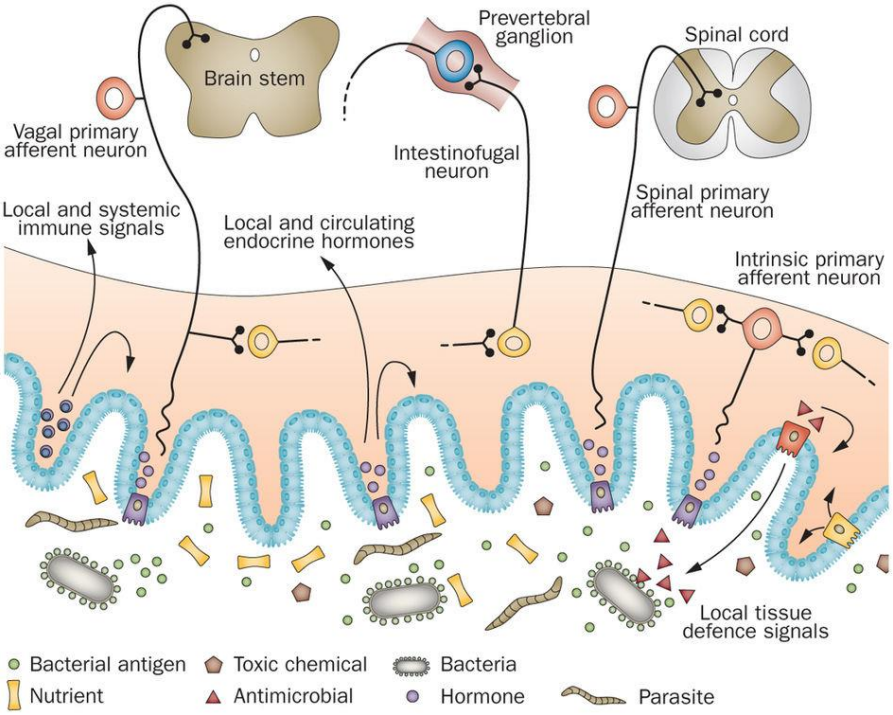


Figure 1.8: Schematic illustrating the intestinal GI tract is subject to local and central innervation. The GI tract responds to nutrients, toxic metabolites, bacteria and hormones by signalling via ENS local signalling pathways or via the CNS. The central signalling pathways include vagal pathways, spinal innervation, and intestinofugal pathways involved in entero-enteric reflexes. Image from Furness *et al.* (2013).

1.7 Gastrointestinal nutrient sensing and the enteroendocrine L cell

The nutrient-rich luminal content is sensed by EECs. In the GI tract, there are at least 10 different types of EECs, that together secrete over 20 different peptides (Polak et al., 1971; Pearse and Bussolati, 1972; Buffa et al., 1975; Polak et al., 1975; Buffa et al., 1976; Alumets et al., 1977; Helmstaedter et al., 1977; Böttcher et al., 1984; Lewin et al., 1998; Date et al., 2000) (Table 1.2). Together, these peptides coordinate complex physiological responses and are critical regulators of GI function and homeostasis (Mace et al., 2015).

EEC	Peptide(s)	Location(s)	Major function of peptide(s)
X/A	Ghrelin	stomach	Food intake, appetite control
D	SOM	stomach, small intestine	Gastrin release, inhibit insulin release
G	Gastrin	stomach (pyloric antral)	Gastric acid release
I	Cholecystokinin (CCK)	proximal small intestine	Gallbladder contraction, stimulate release of pancreatic enzymes
K	Gastric inhibitory peptide (GIP)	proximal small intestine	Insulin release
L	GLP-1 and 2 (GLP-2), PYY, Neurotensin (NTS), Oxyntomodulin (OXM)	distal small intestine, colon	GI motility, reduce food intake, stimulates insulin release, slows gastric emptying
M	Motilin	small intestine	Regulation of MMC and motility
N	NTS	distal small intestine, colon	Gastric acid secretion, intestinal mucosal growth
P	Leptin	stomach	Food intake
S	Secretin	proximal small intestine	Bicarbonate release, gastric acid secretion

Table 1.2: The various types of EECs in the GI tract. The table shows the different types of EECs in the GI tract and the various peptides they each secrete. It also includes the location(s) of each EEC and the major functions of their released peptides. Table from Mace *et al.* (2015).

Together, these various EECs (listed in Table 1.2) constitute <1 % of the total GI mucosal epithelial population and are distributed individually throughout the mucosal epithelium, surrounded by absorptive enterocytes (Buffa et al., 1978). This interspersed nature of EECs is attributed to the lateral inhibition of adjacent cells differentiating into EECs, via a Notch signalling pathway (Schonhoff et al., 2004). The morphology of most EECs in Table 1.2 (with the exception of ghrelin-containing

cells) are open-type, cone-shaped and their microvilli project optimally into the lumen, to achieve maximal nutrient sensing (Shakhlamov and Makar', 1985). In contrast, the ghrelin-containing X/A cells are closed type and unable to sense luminal macronutrients. These X/A cells are activated from the underlying serosal side (paracrine or neurocrine) and subsequently release ghrelin, a powerful appetite stimulant in mouse (Asakawa et al., 2001) and human (Wren et al., 2001).

One of the most relevant EEC cells of interest in anti-diabetic and anti-obesity therapeutics is the L cell. The latter is attributed to the anorectic peptides (namely PYY and oxyntomodulin (OXM)) and the incretin peptide (GLP-1) secreted by this cell type (Albrechtsen et al., 2014). The L cell has an open type morphology and serosal basal processes (Shakhlamov and Makar', 1985). These basal processes have recently been termed neuropods (Bohórquez et al., 2014; Bohórquez et al., 2015). In their study, neuropods projected from the L cell and were escorted by enteric glia to nerve fibre neurite axonal projections. These findings potentially established direct communication between the L cell and the surrounding enteric and CNS neurons (Bohórquez et al., 2014; Bohórquez et al., 2015). In mammals, the frequency of L cells increases along the length of the GI tract, with a preponderance of L cells observed in the distal colon (Figure 1.9) (Sundler et al., 1993). L cells located in the colon co-secrete PYY, GLP-1, GLP-2, and OXM (Böttcher et al., 1984; Böttcher et al., 1986; Cho et al., 2015). Additionally, peptide mRNA encoding gastric inhibitory polypeptide (GIP), cholecystokinin (CCK) and neurotensin (NTS) (Egerod et al., 2012; Habib et al., 2012) has also been identified in small intestinal L cells. The murine L cell has been described as glucose-sensitive (Reimann et al., 2008). Therefore, selective activation of the L cell could protect against hypoglycaemia, unlike current T2DM therapeutics i.e. sulphonylureas (van Staa et al., 1997; Stahl and Berger, 1999; Holstein et al., 2001).

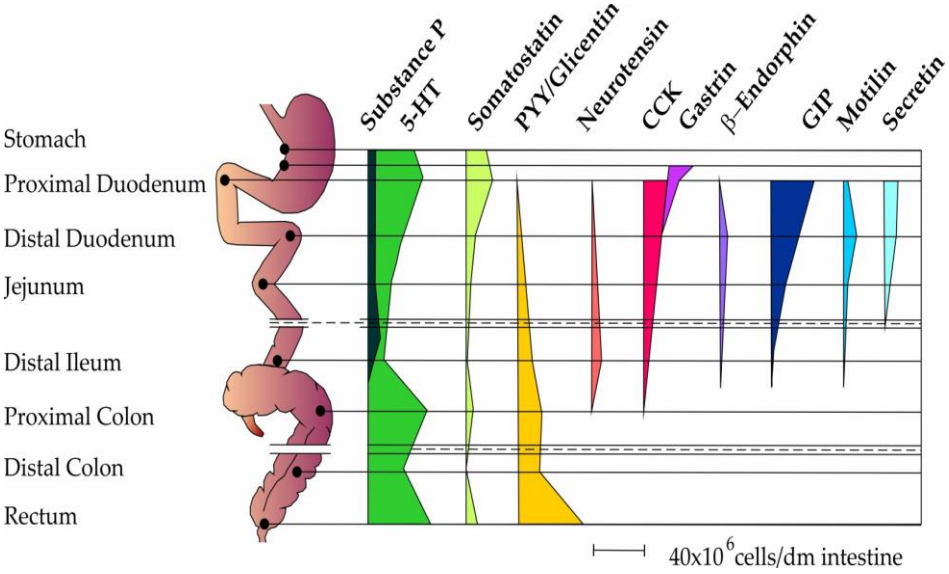


Figure 1.9: Endocrine/paracrine cell types, their peptide localisation and distribution in the mammalian GI tract. The width of the bars represents the density of each cell population. Enteroendocrine L cells (yellow) increase in abundance along the length of the colon, with highest abundance in the descending colon and rectum (adapted from Sundler *et al*. (1993) by Tough IR).

1.7.1 RYGB surgery increases postprandial PYY and GLP-1 plasma concentrations and therefore the L cell is an attractive anti-obesity and anti-diabetic target

RYGB bypass surgery has been classified as the most common and efficacious treatment option of obesity (Smith et al., 2008). The alterations in GI peptides observed postprandially after surgery has been attributed to the successful weight loss and the improvement in glucose tolerance seen in patients (Miras and le Roux, 2013). Before surgery, obese individuals display a blunted postprandial PYY and GLP-1 response, whereas patients who have undergone RYGB surgery, present with postprandial plasma PYY and GLP-1 levels that are vastly superior to lean controls (Figure 1.10) (Borg et al., 2006; le Roux et al., 2006). Well before successful weight loss is achieved post-surgery, patients exhibit an improvement in insulin resistance, insulin production and GLP-1 responses, which may be implicated in the observed T2DM remission and the cessation of anti-diabetic medication (Pournaras et al., 2010). Pories *et al.* (1995) revealed that out of 298 obese individuals with pre-existing T2DM, 91 % of these subjects achieved an improvement in glucose tolerance after RYGB

(Pories et al., 1995). Therefore, it has been postulated that the successful weight loss and improvement in glucose tolerance observed post-RYGB, may be attributed to the increase in L cell derived peptides, GLP-1 (Peterli et al., 2009) and PYY (Korner et al., 2006; Karamanakos et al., 2008) postprandially (le Roux et al., 2006a; le Roux et al., 2007).

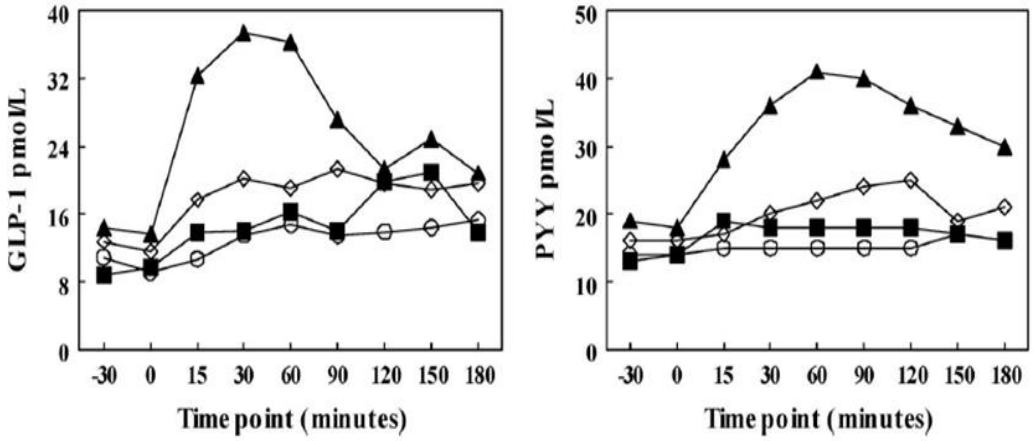


Figure 1.10: Post-RYGB surgery alterations in postprandial GLP-1 and PYY plasma concentrations (pmol/L). The GLP-1 (pmol/L) and PYY (pmol/L) responses to a test meal (420 kcal) in humans who have undergone RYGB (▲, *n*=6), or gastric band (■, *n*=6) compared with lean (◊, *n*=15), and obese controls (O, *n*=12) (le Roux et al., 2006a).

To emulate post-RYGB PYY and GLP-1 alterations, L cell signalling mechanisms have been investigated with selective agonists. Specifically, agonism of L cell-enriched lipid sensing G-protein coupled receptors (GPCRs) including, GPR119 (Overton et al., 2006; Chu et al., 2007; Chu et al., 2008; Cox et al., 2010; Patel et al., 2014), FFA1 (Itoh et al., 2003; Edfalk et al., 2008) and FFA4 (Hirasawa et al., 2005). These GPCRs vary in expression patterns along the length of the GI tract, and are responsible for sensing lipid metabolites in the lumen (Spreckley and Murphy, 2015). Lipid are powerful stimulants of L cell secretion via activation of FFA1, FFA4 and GPR119 (Ekberg et al., 2016). Activation of these GPCRs release L cell-derived peptides, that together have the ability to regulated whole body glucose, increase

insulin release, modulate intestinal mucosal epithelial integrity, slow GI motility and enhance central satiety and appetite (Itoh et al., 2003; Hirasawa et al., 2005; Overton et al., 2006; Chu et al., 2007; Chu et al., 2008; Edfalk et al., 2008; Engelstoft et al., 2008; Cox et al., 2010). At the start of this PhD, in comparison to GPR119, little was known about the pharmacology, functional significance, L cell signalling mechanisms and intestinal and colonic motility effects of FFA1 and FFA4 agonism in the mouse. Knowing FFA1, FFA4 and GPR119 were enriched in L cells, agonism of these three receptors was interrogated with novel selective compounds (synthesised by AstraZeneca (AZ)) to investigate L cell signalling mechanisms.

1.8 The FFA1 (GPR40) receptor

In 2003, GPR40 was deorphanised as the first G-protein coupled FFA receptor (Itoh et al., 2003), which was activated by medium-long chain saturated and unsaturated FFAs (MCFA and LCFA, respectively) (Briscoe et al., 2003; Itoh et al., 2003; Kotarsky et al., 2003). Briscoe *et al.* (2003) demonstrated in human embryonic kidney (HEK) 293 cells expressing GPR40, measuring intracellular calcium ($[Ca^{2+}]_i$), that a range of medium-long chain saturated and unsaturated FFAs (with a carbon length chain greater than 6) activated GPR40. This included linolenic acid, linoleic acid, eicosapentaenoic acid, palmitic acid, oleic acid, pentadencanoic acid and docosahexaenoic acid (DHA) (all $EC_{50s} > 1$ μM). The potency of saturated FFAs (not unsaturated FFAs) was dependent on chain length, with a carbon chain less than 10 or greater than 14, showing little activity (Briscoe et al., 2003). Later in 2008, GPR40 was renamed FFA1 (Stoddart et al., 2008). The mouse FFA1 gene encoded a class A, rhodopsin GPCR, which has 83 % homology to the human FFA1 receptor (Table 1.3).

The expression of FFA1 was first identified in rat and mouse pancreatic β cells (Itoh et al., 2003). Here, FFA1 activation results in glucose-stimulated insulin secretion (GSIS) (Itoh et al., 2003). Later, FFA1 was also identified in human pancreatic islets (Tomita et al., 2005). As a result, soon after FFA1 was deorphanised, it was fast-tracked as a relevant therapeutic target for T2DM. Since its discovery in β cells, FFA1 expression has also been identified in mouse enteroendocrine L cells (Edfalk et al., 2008), CCK-secreting I cells (Liou et al., 2011; Sykaras et al., 2012), GIP-secreting K cells (Parker et al., 2009), pancreatic α cell islets (Flodgren et al., 2007) and taste cells

(Cartoni et al., 2010). More recently, FFA1 has also been found in the CNS. In one study, utilising *in situ* hybridisation, FFA1 mRNA was identified in mouse hippocampal and motor cortex neurons (Zamarbide et al., 2014), whereas in another study, immunohistochemical analysis revealed FFA1 expression in the mouse spinal dorsal horn and dorsal root ganglion neurons (Karki et al., 2015). FFA1 expression in the brain appears to be involved in descending pain pathways (Karki et al., 2015; Nakamoto et al., 2015).

	Mus Musculus	**Rattus Norvegicus**		**Homo-Sapiens**	
	αα	αα	% similarity to mouse	αα	% similarity to mouse
GPR119	335	468	96	335	82
FFA1	300	300	96	300	83
FFA4	361	361	98	Short isoform: 361 Long isoform: 377	86 83

Table 1.3: The total number of amino acids (αα) in the GPR119, FFA1 and FFA4 receptor sequences. The table includes the % of amino acids conserved between Mus Musculus (mouse), Rattus Norvegicus (rat) and Homo-Sapien (human) (created using BLASTp, www.blast.ncbi.nlm.nih.gov).

1.8.1 FFA1 signal transduction in enteroendocrine L cells and pancreatic β cells

Since the deophanisation of FFA1, many studies have interrogated FFA1 signalling mechanisms in various species and assays. The initial FFA1 study demonstrated that activation of mouse/human FFA1 expressed in Chinese Hamster Ovary (CHO) cells or pancreatic β MIN6 cells, resulted in a classic $G_{\alpha q/11}$-mediated Ca^{2+} response (Itoh et al., 2003). Since then, FFA1-mediated $G_{\alpha q}$-signalling has been displayed in many different studies utilising various assays (Briscoe et al., 2003; Briscoe et al., 2006; Christiansen et al., 2008; Negoro et al., 2010). In the L cell, the $G_{\alpha q}$-protein dissociates and activates phospholipase C_{β} (PLC). Hydrolysis of the membrane lipid

phosphatidylinositol bisphosphate (PIP_2) yields inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 immobilises calcium ions (Ca^{2+}) from the endoplasmic reticulum (ER) and this triggers the release of secretory vesicles, containing L cell-derived peptide hormones. Simultaneously, DAG activates protein kinase C (PKC) and protein kinase D1 (PKD) stimulating F-active remodelling, which contributes to the release of L cell secretory vesicles (Figure 1.11) (Edfalk et al., 2008). Two independent studies have linked FFA1 activation to GLP-1 release *in vivo*. First, Edfalk *et al.* (2008) revealed loss of plasma GLP-1 release in $FFA1^{-/-}$ mice, in response to a high fat diet (HFD), compared to the control (Edfalk et al., 2008). Later, Xiong *et al.* (2013) also revealed loss of GLP-1 release to corn oil (containing linolenic and linoleic acid) in $FFA1^{-/-}$ mice (Xiong et al., 2013). Together, these studies confirm that L cell FFA1 activation contributes to GLP-1 release in mice *in vivo.*

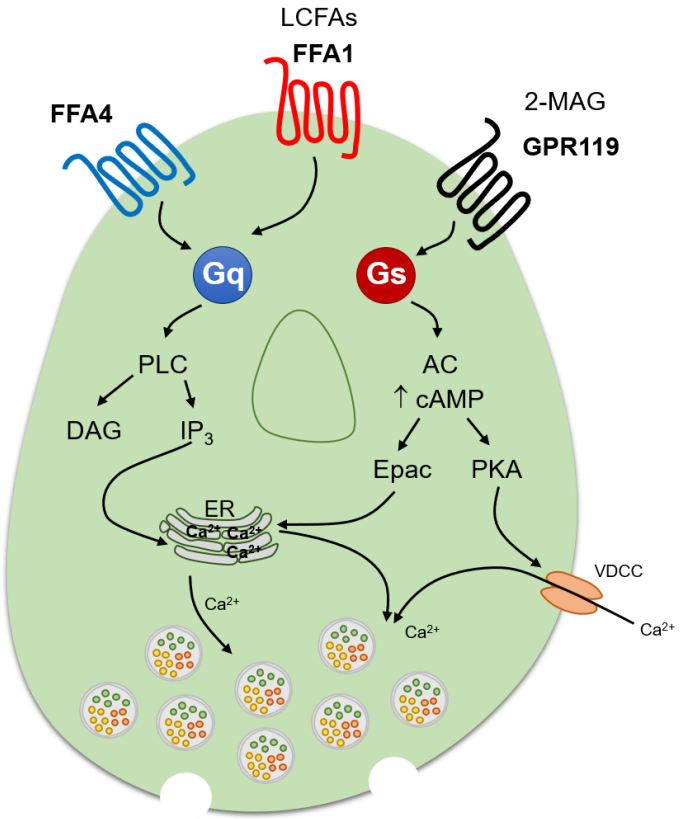


Figure 1.11: Schematic of L cell signalling of FFA1, FFA4 and GPR119. FFA1 and FFA4 signal via the $G_{\alpha q}$ pathway. Activation of this signalling pathway stimulates PLC, which in turn yields IP_3 and DAG. IP_3 activates IP_3 receptors (not shown) expressed in the ER, which increases $[Ca^{2+}]_i$ and causes consequent peptide release. DAG activates PKC/PKD, which contributes to peptide release. GPR119 is $G_{\alpha s}$-

coupled and activates adenylate cyclase (AC), increasing cyclic adenosine monophosphate (cAMP), which activates protein kinase A (PKA) and the exchange protein directly activated by cAMP (Epac), resulting in the release of secretory vesicles within L cells. Image adapted from Hauge *et al.* (2015).

As plasma glucose rises postprandially, glucose is taken up by the β cell (via GLUT2) and this initiates glycolysis. This mechanism creates adenosine triphosphate (ATP) and causes the closure of ATP-sensitive potassium channels (K_{ATP}), depolarising the β cell. This depolarisation causes the influx of Ca^{2+} via L-type voltage dependent calcium channels (VDCC), which consequently results in insulin release (Fu et al., 2013) (Figure 1.12). GSIS is augmented by activation of β cell FFA1 $G_{\alpha q/11}$ PLC pathway (Fujiwara et al., 2005; Shapiro et al., 2005), leading to phosphorylation of PKD1 (Ferdaoussi et al., 2012) and insulin release (Latour et al., 2007; Tan et al., 2008) (Figure 1.12). FFA1 agonism has been shown to amplify β cell GSIS in the presence of high glucose (11 mM and 22 mM) compared to low glucose (5.5 mM), in a MIN6 cell line transfected with FFA1. This indicated FFA1-induced GSIS was glucose-sensitive (Itoh et al., 2003). In another study, Schnell *et al.* (2007), revealed that numerous saturated FFAs and mono- and polyunsaturated FFAs increased the mobilisation of $[Ca^{2+}]_i$ and caused an influx of Ca^{2+} via VDCC in primary mouse β cells, classic characteristics of $G_{\alpha q}$-signalling. This mechanism was abolished in the presence of a specific-FFA1 small interfering ribonucleic acid (siRNA), confirming that this signalling pathway was FFA1-dependent in primary mouse β cells (Schnell et al., 2007).

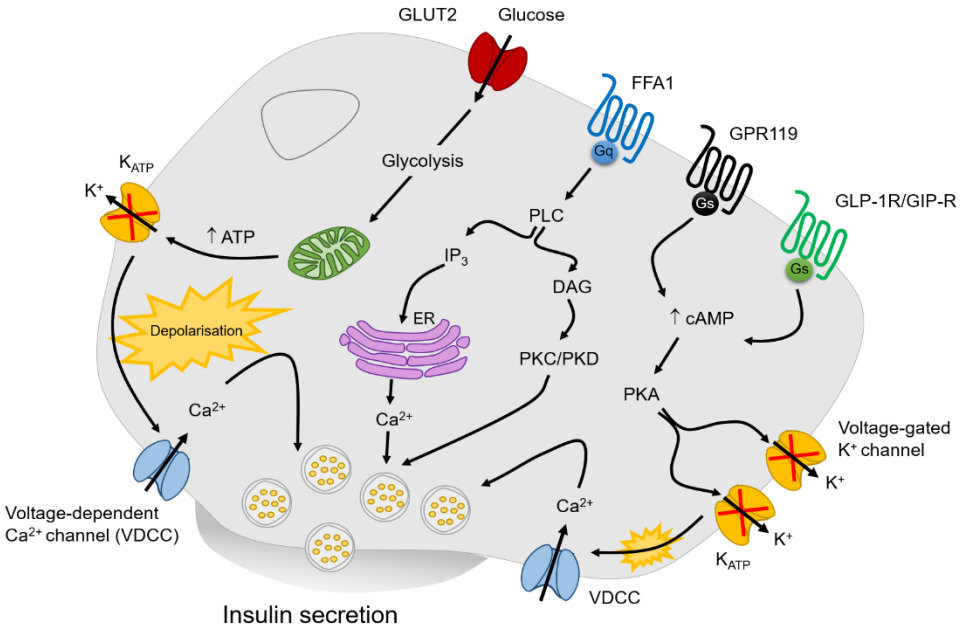


Figure 1.12: Schematic showing various mechanisms of β cell insulin secretion. As the glucose levels rise after a meal, glucose is transported into the pancreatic β cell via the GLUT2 glucose transporter. The cell undergoes glycolysis which releases ATP. The rise in ATP shuts ATP-sensitive potassium channels (K_{ATP}), depolarising the β cell, which causes an influx of Ca^{2+} via VDCC. This rise in $[Ca^{2+}]_i$ triggers insulin secretion. The β cell is enriched with FFA1 and GPR119 receptors that augment insulin secretion via $G_{\alpha q}$ and $G_{\alpha s}$ signalling pathways, respectively. Activation of the GLP-1R and the gastric inhibitory polypeptide receptor (GIP-R) with GLP-1 and GIP respectively, also enhances insulin release via a $G_{\alpha s}$-stimulatory pathway. Image adapted from Li *et al.* (2016).

1.8.2 FFA1 polymorphisms

The polymorphism Arg211His was identified in the FFA1 gene as a missense variant (Haga et al., 2002). A study carried out in healthy Japanese men demonstrated that subjects with the homozygous Arg/Arg variant, showed decreased serum insulin, β cell function and basal insulin sensitivity. In the same study, there were no clear differences observed in genotype and allele frequency between the healthy population and a T2DM cohort. This study concluded that this gene variant may contribute to a variation in insulin secretion capacity, which could ultimately lead to β cell dysfunction and T2DM. However, further *in vitro* and *in vivo* experimentation was

advised (Ogawa et al., 2005). On the contrary, Hamid *et al.* (2005) revealed that the Arg211His polymorphism does not contribute to dysregulation of FFA1 signalling *in vitro* or insulin secretion in healthy and T2DM volunteers (Hamid et al., 2005). Two other rare FFA1 polymorphisms have been discovered, Asp175Asn (Hamid et al., 2005) and Gly180Ser (Vettor et al., 2008). Gly180Ser and Asp175Asn caused a decrease in receptor function in HeLa cells (measuring oleic-induced increase in $[Ca^{2+}]_i$) (Vettor et al., 2008) and COS-7 cells (measuring 5,8,11-eicosatriynoic acid-induced [^{3}H]-myo-inositol turnover), respectively (Hamid et al., 2005). However, thorough pharmacological investigations undertaken by Smith *et al*. (2009) failed to observe any functional difference between these three polymorphic variants (Arg211His, Gly180Ser and Asp175Asn) expressed in HEK293 cells (Smith et al., 2009). The inconsistencies indicate that there were no clear findings to suggest that these FFA1 polymorphisms were detrimental to FFA1 receptor function.

1.8.3 FFA1$^{-/-}$ studies

In 2003, Itoh *et al.* demonstrated that 50 % of the FFA-induced GSIS response in MIN6 cells was abolished in the presence of FFA1 siRNA *in vitro*, indicating FFA1 agonism was a major contributor to FFA-induced GSIS (Itoh et al., 2003). The first study to utilise FFA1$^{-/-}$ mice, showed these mice were protected against obesity-induced hyperglycaemia, glucose intolerance, hyperinsulinemia, fatty liver development, increased hepatic glucose and hypertriglyceridemia (Steneberg et al., 2005). In the same study, overexpression of FFA1 in mice, impaired β cell function, caused hypoinsulinemia and resulted in diabetes *in vivo* (Steneberg et al., 2005). In this way, an FFA1 antagonist appeared to be advantageous as a therapeutic for obesity and diabetes. Conversely, Latour *et al.* (2007) showed that FFA1$^{-/-}$ mice have normal glucose tolerance and insulin secretion in response to a glucose stimulus. However, the insulin response to Intralipid (fat emulsion) was attenuated by 50 %. This suggested that 50 % of the GSIS response was FFA1-mediated (Latour et al., 2007), as shown previously (Itoh et al., 2003). Therefore, an FFA1 agonist was proposed as a potential anti-T2DM therapeutic. Similarly, Lan *et al.* (2008) and Alquier *et al.* (2009) revealed 50 % and 60 % loss of the GSIS response respectively, in FFA1$^{-/-}$ mice *in vivo*. Additionally, Lan *et al*. (2008) demonstrated that when these FFA1$^{-/-}$

mice were introduced to a HFD (60 % calories from fat), they were not protected against the adverse effects associated with a HFD. Both wild-type (WT) and FFA1$^{-/-}$ mice were obese, developed insulin resistance and a fatty liver (Lan et al., 2008). This suggested loss of FFA1 does not provide protection against HFD. Similarly, Kebede *et al.* (2008) showed that FFA1$^{-/-}$ mice fed a HFD (60 % for 11 weeks) also displayed fasting hyperglycaemia, became obese, glucose intolerant and insulin resistant with associated fatty liver disease, and their insulin secretory responses were blunted compared to WT (Kebede et al., 2008). Notably, Nagasumi *et al.* (2009) demonstrated that overexpression of human FFA1 in pancreatic β cells improved glucose tolerance and enhanced GSIS in mice, in an oral glucose tolerance test (OGTT). As FFA1 agonism stimulates GSIS (Itoh et al., 2003) and 50-60 % of this response was abolished in FFA1$^{-/-}$ mice *in vivo* (Latour et al., 2007; Lan et al., 2008; Alquier et al., 2009), a selective FFA1 agonist rather than a FFA1 antagonist might be beneficial in future T2DM therapeutics.

1.8.4 FFA1 agonists

Since the deorphanisation of FFA1, many FFA1 agonists have been synthesised to interrogate FFA1 signalling mechanisms in various assays. In this next section, only the FFA1 agonists (TAK-875, JTT and TUG424) and the dual FFA1 and FFA4 agonists (GW9508 and pinolenic acid) used in this thesis will be described.

TAK-875 (produced by Takeda) also known as Fasiglifam (Negoro et al., 2010; Negoro et al., 2012a; Negoro et al., 2012b), was the first FFA1 agonist to enter clinical trials as an orally available, potent and selective agonist. Pre-clinical studies demonstrated TAK-875 enhanced GSIS in rat and human pancreatic islets *in vitro* (Yashiro et al., 2012). Furthermore, TAK-875 (at 10 mg/kg) elevated plasma insulin and fasting hyperglycaemia in male Zucker T2DM rats *in vivo*, without the risk of hypoglycaemia (Tsujihata et al., 2011). Attributed to the success of TAK-875 in preclinical studies, this agonist progressed into clinical development. The first Phase I clinical trial in healthy volunteers showed TAK-875 was well tolerated and rapidly absorbed (Naik et al., 2012). In a Phase II study, multiple ascending doses in diabetic volunteers, improved their glucose tolerance without dose-related adverse effects (Leifke et al., 2012). The success of Phase I and II studies allowed TAK-875 to

proceed to phase III clinical trials. A phase III trial in T2DM Japanese patients found TAK-875 significantly reduced glycosylated haemoglobin A1c (HbA1c, measurement of blood glucose over 2-3 months) compared to the placebo, improved glycaemic control and possessed a minimal risk of hypoglycaemia (Kaku et al., 2015). Unfortunately, in Phase III trials TAK-875 induced liver toxicity and therefore the clinical trial was terminated. TAK-875 was resynthesised by AZ and this selective FFA1 agonist was used in this thesis.

Recently, the crystal structure of the FFA1 receptor was discovered utilising the selective FFA1 ligand, TAK-875. TAK-875 bound the FFA1 receptor via the lipid bilayer, between the seven-transmembrane helix 3–5 and the extracellular loop 2. The carboxylate of TAK-875 interacted with two critical residues, Arg^{183} and Arg^{258}, within the binding pocket. A third important residue, Asn^{244} formed a hydrogen bond with Arg^{258}. Once TAK-875 bound to the FFA1 receptor, it protruded outside of transmembrane domains III and IV (Srivastava et al., 2014). An earlier study suggested there may be multiple binding sites within the FFA1 receptor, including two allosteric sites and one orthosteric site (Lin et al., 2012). TAK-875 and most other synthetic FFA1 agonists appear to bind the FFA1 allosteric site, and their carboxylate tails interact with the two Arg residues (Hassing et al., 2016b). Indeed, TAK-875 has been termed a partial agonist on its own and a positive allosteric modulator when used in combination with γ-linolenic acid, resulting in robust amplification of GSIS in mouse pancreatic islets (Yabuki et al., 2013).

JTT-851 was synthesised by Japan Tobacco. This FFA1 agonist progressed to clinical trials and in 2013 the phase II study (NCT01699737) was completed. Since 2013, no further development has been reported. JTT-851 was resynthesised by AZ, as a selective and potent FFA1 agonist and henceforth it will be referred to as JTT.

TUG424, a potent FFA1 full agonist (compound 20; (Christiansen et al., 2008)) from the alkyne agonist series, significantly increased GSIS (2-fold) in a glucose-dependent manner, in a rat insulin-secreting β cell line (INS-1E) and in islets from WT mice. This elevation in insulin secretion was absent in islets retrieved from $FFA1^{-/-}$ mice, suggesting the GSIS effect was FFA1-mediated (Christiansen et al., 2008). TUG424, which is commercially available was used in this study, as a selective FFA1 agonist.

1.8.5 Dual FFA1 and FFA4 agonists

GW9508 was discovered during a GlaxoSmithKline (GSK) chemical collection in 2006, and was marketed as a dual FFA1 and FFA4 agonist (Briscoe et al., 2006). Notably, GW9508 had a 100-fold greater selectivity and a 70-fold higher potency for FFA1 than FFA4. Due to the lack of FFA1 selective agonists in many of the initial FFA1 studies, GW9508 was the preferred agonist of choice. In the initial studies, GW9508 increased $[Ca^{2+}]_i$ and promoted glucose-dependent GSIS in FFA1 or FFA4 transiently transfected HEK293 cells and MIN6 insulinoma cells, respectively (Briscoe et al., 2006). GW9508, which is commercially available was used in this study as a dual FFA1 and FFA4 agonist.

Pine nut oil (PNO) from the Korean pine (*Pinus koraiensis*), is commonly used in food preparation and condiments worldwide. PNO is composed of 92 % poly- and monounsaturated FFAs including oleic acid, linolenic acid and pinolenic acid (Xie et al., 2016). Pinolenic acid constitutes 15-20 % of PNO and has recently been identified as a FFA1 and FFA4 dual agonist (Christiansen et al., 2015). In the past, many studies have examined the beneficial effects of PNO. In these studies, PNO reduced weight gain and intramuscular lipid accumulation in mice (Lee et al., 2004); provided protection against hypercholesterolaemia, thrombosis and hypertension in rats (Sugano et al., 1994) and had apparent appetite suppressant effects in postmenopausal women, which was attributed to an increase in GI peptides, namely GLP-1 and CCK-8 (Pasman et al., 2008). Pinolenic acid, which is commercially available was used in this thesis as a dietary dual FFA1 and FFA4 agonist.

1.8.6 FFA1 antagonists

FFA1 antagonists have limited therapeutic value in T2DM, however they are pertinent pharmacological tools. The first FFA1 antagonist synthesised was GW1100. It selectively inhibited GW9508-stimulated Ca^{2+} elevation and GSIS in HEK293 cells and MIN6 cells transfected with FFA1, respectively (Briscoe et al., 2006). To date, GW1100 is still used as the most common selective commercially available FFA1 antagonist. Over the years, two other FFA1 antagonists have been synthesised, namely ANT203 and ANT825 (also known as compound 39) (Waring et al., 2015). According to AZ, ANT203 and ANT825 exhibit identical pharmacology (D. Smith

and M. Schindler, personal communication). Notably, while acute exposure of FFAs prompt GSIS, chronic exposure of FFAs (i.e. palmitate) are associated with β cell apoptosis, which impairs insulin secretion (Zhou and Grill, 1995). In obese individuals, prolonged elevation of FFAs appears to be linked to insulin resistance and T2DM (Kashyap et al., 2003). Kristinsson *et al*. (2013) demonstrated that the FFA1 antagonist, ANT203, significantly reduced the deleterious effects of palmitate on β cells. This revealed a role for FFA1 in palmitate-induced β-cell apoptosis, but this was dependent on the presence of extracellular FFAs. Thus, Kristinsson *et al.* (2013) concluded that only in the absence of chronically high levels of circulating FFAs was FFA1 agonism advantageous, stimulating GSIS. More recent studies have shown FFA1 agonism (TUG-469, 3 and 10 μM) caused GSIS, protected β cells and abolished palmitate-induced β cell apoptosis in murine islets and rat INS-1E cells (Wagner et al., 2013; Panse et al., 2015). Conversely, the FFA1 antagonist, TUG-761 (10 μM) induced apoptosis and this effect was absent in islets from $FFA1^{-/-}$ mice (Wagner et al., 2013). Taken together, these studies further confirm FFA1 agonism may be advantageous for future T2DM therapeutics. Recently, the FFA1 antagonist, ANT825 (80 mg/kg) reduced non-esterified fatty acid (NEFA)-induced insulin secretion by approximately 70 %, in male insulin resistant Zucker fa/fa rats. Thus, while FFA1 antagonists are not beneficial as T2DM therapeutics, they may be valuable in the treatment of hyperinsulinemia or insulin resistant states, where insulin levels are elevated due to GSIS (Waring et al., 2015). The commercially available FFA1 antagonist, GW1100 and the selective AZ supplied FFA1 antagonist, ANT825 were use in this PhD study.

1.9 The FFA4 (GPR120) receptor

GPR120, now designated FFA4 (Davenport et al., 2013) was isolated from human genomic deoxyribonucleic acid (DNA) fragments and deorphanised in 2005, as the second FFA GPCR for LCFAs (Hirasawa et al., 2005). FFA4, a seven-transmembrane class A rhodopsin-like receptor is activated by a range of medium-long chain saturated and polyunsaturated fatty acids (C12-C22; particularly omega-3 and omega-6 FFAs) (Hirasawa *et al.*, 2005).

Initial FFA4 studies revealed FFA4 mRNA expression was abundant in mouse and human intestine, lung (Hirasawa et al., 2005; Miyauchi et al., 2009; Little et al., 2014; Mizuta et al., 2015) and adipose (Gotoh et al., 2007; Miyauchi et al., 2009). Hirasawa and colleagues also demonstrated that FFA4 mRNA was colocalised with GLP-1 in GLP-1-producing human colonic cells i.e. enteroendocrine L cells, utilising *in situ* hybridisation and a GLP-1 antibody. This was confirmed using reverse transcription polymerase chain reaction (RT-PCR), which showed an abundance of FFA4 mRNA in GLP-1 positive cells and a minor detection of FFA4 mRNA in GLP-1 negative cells (Hirasawa et al., 2005). Since this study, the FFA4 receptor has been widely expressed in intestinal enteroendocrine K cells (Iwasaki et al., 2015), I cells (Sykaras et al., 2012), gastric ghrelin cells (Koyama et al., 2016) and gastric somatostatin cells (Egerod et al., 2015). FFA4 expression has also been identified in murine pancreatic delta cells (Stone et al., 2014), clonal β cells (Moran et al., 2014), immune macrophages (Oh et al., 2010), taste cells (Cartoni et al., 2010) and in hypothalamic microglia (Dragano et al., 2017). In this way, FFA4 is more widely expressed compared to the FFA1 receptor.

There are two splice variants of the human FFA4 receptor, a short isoform (GPR120S) and a long isoform (GPR120L), while only one variant exists in rodents and cynomolgus monkey. The human GPR120S is homologous to the FFA4 receptor found in rodents and cynomolgus monkey (Moore et al., 2009), and 86 % of its amino acid sequence is conserved when compared with the mouse FFA4 receptor sequence (Table 1.3). Notably, the GPR120L has an insertion of 16 amino acids in the third intracellular loop and this region of the long isoform, is important for G-protein and β-arrestin recognition and association. Recently, the differential signalling of the two human isoforms was investigated in HEK293 cells (Watson et al., 2012). Watson *et al.* (2012) revealed that activation of the short isoform (GPR120S) with the dual FFA1 and FFA4 synthetic agonist, GW9508 increased $[Ca^{2+}]_i$ (EC_{50} 3.2 µM (1.6 – 6.3)) and also recruited β-arrestin 2. Conversely, activation of the long isoform (GPR120L) recruited β-arrestin 2 but was unresponsive in the Ca^{2+} mobilisation assay, identifying signalling bias between the two human FFA4 isoforms (Watson et al., 2012). Further studies are required to understand the importance of the FFA4 long isoform in humans. In most human FFA4 studies in the literature, the GPR120S isoform has been utilised

(Suckow and Briscoe, 2016), and therefore these studies are comparable with murine FFA4 receptor studies.

The expression of FFA4 varies between lean and obese humans. As BMI increased, the expression of duodenal FFA4 was upregulated (Little et al., 2014). This corresponded to data from Paulsen *et al.* (2014), which showed FFA4 expression was elevated in the distal ileum in diet-induced obese (DIO) rats, when compared with diet-resistant (DR) rats (Paulsen et al., 2014). Conversely, Fam *et al.* (2015), revealed a decrease in FFA4 expression in DIO mouse ileum (Fam et al., 2015). This suggests FFA4 expression may be regulated differently in different DIO rodents (Paulsen et al., 2014; Fam et al., 2015). Nevertheless, the upregulation of FFA4 in DIO rat ileum (Paulsen et al., 2014) was more comparable with changes observed in human (obese) duodenum (Little et al., 2014).

1.9.1 FFA4 signal transduction in enteroendocrine L cells

The first FFA4 study showed that α-linolenic acid stimulated a rise in $[Ca^{2+}]_i$ in a concentration-dependent manner, in HEK293 cells stably expressing the murine GPR120-Gα16 fusion protein. In the same study, FFA4 agonism had no effect on cAMP, therefore indicating that FFA4 activation was $G_{\alpha q}$-mediated and does not couple to $G_{\alpha s}$ (Hirasawa et al., 2005). Thus, in enteroendocrine L cells FFA4 agonism generates IP_3 and DAG, which stimulate the release of L cell-derived peptides (Figure 1.11). Many *in vitro* studies have recapitulated FFA4-$G_{\alpha q}$ signalling revealing an increase in $[Ca^{2+}]_i$ (Hara et al., 2009; Watson et al., 2012; Hudson et al., 2013). More recently the promiscuity of FFA4 signalling was revealed, with reports showing FFA4 couples to the $G_{\alpha i}$-protein in mouse gastric somatostatin cells (Egerod et al., 2015), gastric ghrelin A/X cells (Koyama et al., 2016) and pancreatic somatostatin delta cells (Stone et al., 2014). In this way, FFA4 agonism in different EEC types leads to the association of different G-proteins.

1.9.2 FFA4 signal transduction in macrophages

In macrophages, activation of the toll-like receptor (TLR) 4 by lipopolysaccharides (LPS) and activation of a second receptor, tumour necrosis factor α receptor (TNFR) by tumour necrosis factor (TNF) α, cause activation of pro-inflammatory signalling (Figure 1.13, right side). Activation of FFA4 leads to receptor phosphorylation by GPCR kinases. The phosphorylated FFA4 binds β-arrestin 2 which induces internalisation of the FFA4-β-arrestin 2 complex. This complex binds TGF-β activated binding protein 1 (TAB1), which prevents the formation of a complex consisting of TAB1 and transforming growth factor β (TGF-β) activated kinase 1 (TAK1). This ultimately inhibits downstream pro-inflammatory pathways involving I_KB kinase (IKK)/ nuclear factor (NF)-KB and mitogen-activated protein kinase kinase 4 (MKK4)/c-Jun NH_2-terminal kinase (JNK) (Oh et al., 2010; Oh et al., 2014). This pro-inflammatory pathway was inhibited by the selective FFA4 agonist, TUG891 (IC_{50} 1.4 μM (0.7 – 2.7)) in murine RAW264.7 macrophages (Hudson et al., 2013). Li *et al.* (2013) further interrogated this anti-inflammatory mechanism in RAW264.7 cells and demonstrated that the omega-3 FFA, DHA inhibits cyclooxygenase 2 (COX-2) (downstream of (NF)-KB) and subsequent prostaglandin E_2 synthesis, via a FFA4 mechanism (Li et al., 2013). Unlike FFA4 L cell signalling, the FFA4 mechanism in macrophages appears to be β-arrestin 2 dependent and independent of $G_{\alpha q/11}$ (Oh et al., 2010; Oh et al., 2014).

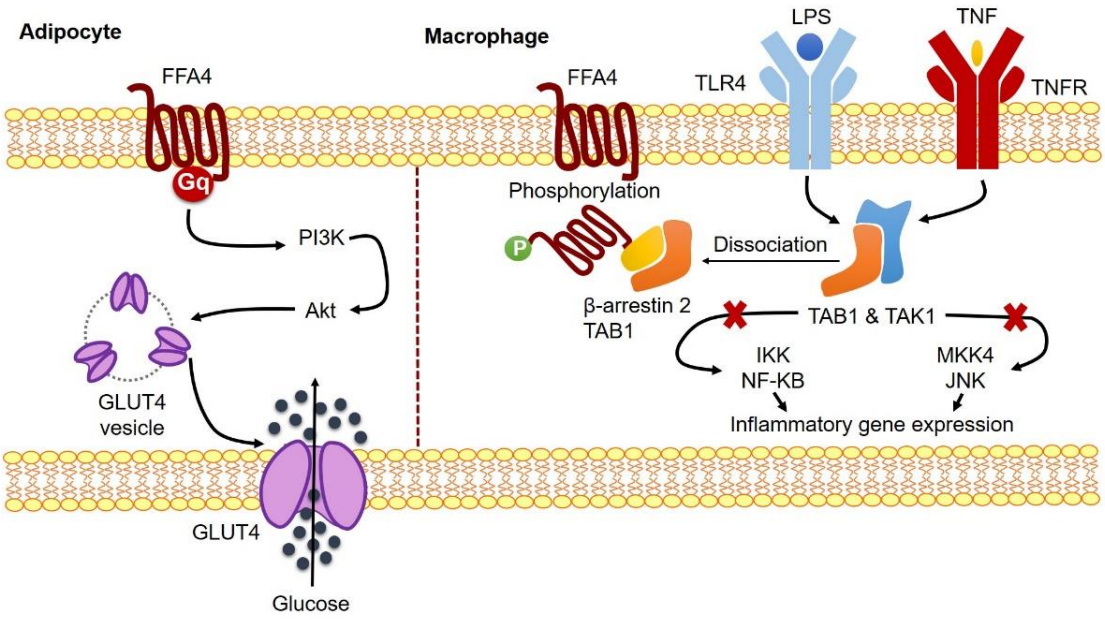


Figure 1.13: Differential FFA4 signalling pathways in adipocytes (left) and macrophages (right). In adipocytes, activation of FFA4 stimulates $G_{\alpha q}$-coupled signalling and leads to the activation of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and a downstream protein kinase B (PKB, also known as Akt) pathway. This increases the glucose transport into adipocytes by inserting GLUT4 into the plasma membrane, which enhances adipogenesis. In macrophages, FFA4 activation leads to phosphorylation of FFA4, and the subsequent binding of β-arrestin 2 causes internalisation of the FFA4 receptor. Image adapted from Li *et al.* (2016).

1.9.3 FFA4 signal transduction in adipocytes

The expression of FFA4 mRNA has been identified in four types of human adipose tissue, namely subcutaneous, perirenal, mesenteric and epididymal tissue (Gotoh et al., 2007). FFA4 mRNA increases during adipocyte differentiation and is strongly expressed in mature adipocytes (Gotoh et al., 2007). Activation of the FFA4 receptor in adipocytes leads to the activation of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and a downstream protein kinase B (PKB; also known as Akt) pathway (Figure 1.13, left side). This results in translocation of vesicles containing the glucose transporter, GLUT4 to the plasma membrane, and this enhances glucose uptake, adipogenesis and insulin sensitivity (Katsuma et al., 2005; Oh et al., 2010; Hudson et al., 2013). In STC-1 cells, the presence of a PI3K inhibitor prevented Akt activation,

and thus FFA4-induced anti-apoptotic effects were abolished (Katsuma et al., 2005). This confirmed the FFA4-induced anti-apoptotic effects were $G_{\alpha q}$-PI3K mediated in adipocytes. Later in 2010, Oh *et al.* demonstrated that the polyunsaturated FFA, DHA enhanced glucose uptake and GLUT4 transport to the plasma membrane. This mechanism was FFA4-, GLUT4- and $G_{\alpha q}$-dependent in 3T3-L1 adipocytes, and was also independent of β-arrestin 2 involvement (Oh et al., 2010). These insulin-sensitising effects of FFA4 agonism (Oh et al., 2010) are thought to contribute to combatting insulin resistance and fasting hyperglycaemia, which are observed in $FFA4^{-/-}$ mice on HFD (Ichimura et al., 2012).

1.9.4 The FFA4 polymorphism

FFA4 receptor dysfunction caused by a single nucleotide mutation (p.R270H) in the long isoform of the receptor (designated R254H in the short FFA4 isoform), has been associated with an increased risk in obesity in humans (European population, minor allele frequency of ~2 %). Expression of this missense non-synonymous mutation, p.R270H in HEK293 cells significantly reduced the elevation of $[Ca^{2+}]_i$ to α-linolenic acid, compared to cells expressing non-mutant FFA4. Furthermore, ALA stimulated GLP-1 release and this was ablated in human intestinal cells (NCI-H716) transfected with the variant p.R270H, compared to the control (Ichimura et al., 2012). This study demonstrated that the presence of the FFA4 variant, p.R270H was not only associated with obesity in humans but also caused FFA4 signalling dysfunction *in vitro*. This low-frequency allele was associated with an increased fasting hyperglycaemia in a European population but was not linked to an increased risk of T2DM (Bonnefond et al., 2015). Recently, Vestmar *et al.* (2016) revealed that the presence of R270H in HEK293 cells also reduced FFA4 surface expression, basal FFA4 constitutive activity and $G_{\alpha q}$ signalling, but did not affect β-arrestin signalling *in vitro*. As FFA4 signalling is $G_{\alpha q}$-mediated in L cells and β-arrestin dependent in macrophages, the lack of $G_{\alpha q}$ but not β-arrestin signalling, implicates a possible risk for intestinal metabolic disruption but not macrophage inflammatory dysfunction. Despite the observed dysfunctional FFA4 signalling *in vitro*, this did not predispose a Danish population to an increase risk of obesity or obesity related metabolic disorders (Vestmar et al., 2016). The presence of R270H/R254H appears to disrupt FFA4 signalling *in vitro*

and *in vivo.* However, further studies are required to determine whether this predisposes individuals to obesity and obesity-related disorders *in vivo*.

1.9.5 FFA4$^{-/-}$ studies

Typically, FFA4$^{-/-}$ mice on a normal chow diet are glucose-intolerant, possess insulin resistance and are obese with increased inflammation (Oh et al., 2010). When introduced to a HFD (60 %, 15-20 weeks), FFA4$^{-/-}$ mice were unable to respond to omega-3 FFAs, whereas WT mice (+HFD) given omega-3 FFAs showed an improvement in glucose tolerance, insulin sensitivity and a reduction in inflammation. This study demonstrated the beneficial insulin-sensitising effects of FFA4 activation (Oh et al., 2010). Ichimura *et al.* (2012) also observed insulin resistance in FFA4$^{-/-}$ mice, as seen previously by Oh *et al.* (2010). Conversely, these mice only displayed glucose intolerance and an increased body weight when on a HFD (60 %), not on normal chow (Ichimura et al., 2012). At odds with these studies, Suckrow *et al.* (2014) demonstrated FFA4$^{-/-}$ mice were hyperglycaemic, hyperinsulinemia, possessed an increased body weight and were glucose-intolerant, but not insulin resistant (Suckow et al., 2014). Together, data from these three studies show clear discrepancies, which have been attributed to possible differences in animal backcrossing, housing or knockout strategies.

1.9.6 FFA4 agonists

The polyunsaturated FFAs, omega-3 and omega-6, are potent activators of FFA4 and are provided by dietary fish oil and supplements. However, the quantity of fish oil that would need to be consumed, to chronically activate FFA4 is impractical and therefore activating FFA4 with a proven, potent small molecule agonist may be more practical and beneficial long term (Hirasawa et al., 2005). The search for FFA4 selective agonists has been challenging, as most endogenous fatty acids are nonselective and activate both FFA1 and FFA4 (Hudson et al., 2011). This is surprising as only 10 % of their amino acid sequences are conserved between these two receptors. Since the discovery of FFA4, many FFA4 agonists have been generated. However, none of these agonists have progressed to clinical development.

The first natural FFA4 ligands, grifolin acid and grifolin methyl ether were isolated from the fruiting bodies of the plant, *Albatrellus ovinu.* However, these grifolin derivatives only provided modest efficacy (Hara et al., 2009). In 2010, the initial lead synthetic FFA4 agonists, including NCG21 (Suzuki et al., 2008), were derived from peroxisome proliferator-activator receptor (PPAR) γ agonists. Unfortunately, these agonists only provided modest selectivity (10-15 fold) and efficacy over FFA1, and micromolar concentrations (μM) were required to induce FFA4 signalling (Sun et al., 2010). Hence, the development of a potent and selective FFA4 agonist was critical to interrogate the pharmacology and function of the FFA4 receptor. In the section that follows, I shall focus on the agonists used in this PhD, namely TUG891, Met-36 and AZ423.

In 2013, TUG891 was described as the first potent and selective agonist for human and murine FFA4 (Shimpukade et al., 2012; Hudson et al., 2013). TUG891 increased $[Ca^{2+}]_i$ in HEK293 cells expressing the human (EC_{50} 117.5 nM (100.0 – 138.0) or mouse FFA4 receptor (EC_{50} 128.8 nM (104.7 – 158.5)). Notably, TUG891 exhibited poor selectivity between the mouse FFA4 and FFA1 receptors (EC_{50} 389.0 nM (323.6 – 467.7)) in a calcium mobilisation assay, indicating TUG891 possibly exerts dual FFA1 and FFA4 agonism, in this species. Furthermore, TUG891 induced β-arrestin 2 recruitment (HEK293T cells) in a bioluminescence resonance energy transfer (BRET) assay, 288-fold and 61-fold more potently at human and mouse FFA4 respectively, compared to the FFA1 receptor. Furthermore, TUG891 possessed only 3-fold selectivity between the FFA4 receptor and the FFA1 receptor, in a Ca^{2+} assay in HEK293 cells. This revealed TUG891 exhibited poor selectivity between murine FFA4 and FFA1 in this assay. In the same study, TUG891 robustly secreted GLP-1 in STC-1 and GLUTag EEC lines, enhanced [3H]deoxyglucose uptake in 3T3-L1 adipocytes and inhibited TNF-α secretion in RAW264.7 macrophages (IC_{50} 1.4 μM (0.7 – 2.7)). Thus, TUG891 enhanced adipogenesis and inhibited pro-inflammatory responses. TUG891 was used in this thesis as a commercially available selective FFA4 agonist (Hudson et al., 2013).

The FFA4 agonists, Metabolex-36 (Ma et al., 2010) and AZ423 (McCoull et al., 2017) are 100-fold selective for FFA4 over FFA1. Stone *et al.* (2014) demonstrated these two agonists inhibited glucose-dependent somatostatin secretion via a FFA4-meditated inhibitory $G_{\alpha i}$-signalling pathway, in pancreatic delta cells. Apart from this

study, there has been limited information regarding the function of Metabolex-36 and AZ423, *in vitro* or *in vivo*. Recently, AZ423 (also known as compound 34, McCoull *et al.* (2017)), significantly reduced glucose excursions after a glucose load, an effect that was absent in FFA4$^{-/-}$ mice (McCoull et al., 2017). Thus, showing for the first time that AZ423 improves glucose tolerance via an FFA4-mediated mechanism. Metabolex-36 was resynthesised by AZ and in this thesis, it has been abbreviated to Met-36.

1.9.7 The FFA4 antagonist

To date, the only commercially available FFA4 antagonist is AH-7614 (previously known as compound 39, Sparks *et al.* (2014)). In 2014, this FFA4 antagonist was discovered as a xanthene derivative of a diarylsulfonamide based FFA4 agonist (Sparks et al., 2014). AH-7614 inhibited the ability of linoleic acid to stimulate $[Ca^{2+}]_i$ in U2OS cells transfected with mouse (IC_{50} 7.9 nM) or human FFA4 (IC_{50} 79.4 nM). In the same study, this FFA4 antagonist inhibited agonist (GSK137647A)-induced GSIS in MIN6 cells and GLP-1 secretion in the human intestinal cell line, NCI-H716 (Sparks et al., 2014). AH-7614 is commercially available and was used in this PhD study as a selective FFA4 antagonist.

1.10 The GPR119 receptor

In 2003, GPR119 was deorphanised using a bioinformatics approach of the human genome. This receptor appeared to be a class A GPCR that did not closely resemble FFA1 or FFA4 (Fredriksson et al., 2003). The GPR119 mouse gene encodes a 335 amino-acid sequence and this has 82 % homology to the human receptor (Table 1.3). Like FFA1, the GPR119 receptor was fast-tracked as a relevant therapeutic target for diabetes and obesity, due to its enrichment in mouse and human pancreas (Chu et al., 2008; Odori et al., 2013) (specifically the islets (Chu et al., 2007)) as well as the GI tract (stomach, duodenum, jejunum, ileum and colon) (Soga et al., 2005; Chu et al., 2007; Chu et al., 2008). Initial studies conducting *in situ* hybridisation analysis revealed GPR119 was expressed in rat pancreatic islets, specifically in β cells (Chu et al., 2007). Additionally, GPR119 mRNA was identified in preproglucagon-

expressing cells of mouse proximal colon i.e. GLP-1 secreting L cells (Chu et al., 2008). This was later confirmed in mouse and human L cell cell-based assays (mouse GLUTags and human NCI-H716) (Lauffer et al., 2009). Furthermore, the expression of GPR119 in the human brain was low, which strengthened the safety profile of this receptor (Soga et al., 2005; Chu et al., 2008). Theoretically, the expression of GPR119 in β cells could increase insulin secretion directly (Figure 1.12), while L cell GPR119 agonism (Figure 1.11) could stimulate the release of the incretin, GLP-1 and thus cause insulin secretion via β cell GLP-1R (Chu et al., 2008) (Figure 1.12). In previously published mouse mucosal studies, GPR119-induced anti-secretory responses that increased along the length of the GI tract, and the greatest response was observed in the descending colon (Cox et al., 2010). This response profile correlated with the initial murine GPR119 mRNA expression pattern (Chu et al., 2008). In humans, the greatest GPR119 mRNA expression was observed in the pancreas (Soga et al., 2005; Chu et al., 2008), followed by the GI tract (Chu et al., 2008). In the human intestine, GPR119 expression was greatest in the duodenum, whereas expression levels in the jejunum, ileum and colon were similar in each region (Chu et al., 2008; Odori et al., 2013). Similarly, Patel *et al.* (2014) revealed human GPR119 expression was uniform along the GI tract, with a slight elevated GPR119 expression level in the duodenum (Patel et al., 2014). The expression of GPR119 receptor in mouse has also been identified in pancreatic polypeptide (PP) cells (Sakamoto et al., 2006), duodenal CCK-I cells (Sykaras et al., 2012), upper small intestine GIP-K cells (Parker et al., 2009) and pancreatic α cells (Odori et al., 2013). In this way, GPR119 and the FFA1 and FFA4 receptors are similarly expressed in intestinal L, K and I cells.

1.10.1 GPR119 endogenous agonists

The GPR119 endogenous ligands are thought to include the ethanolamide derivative, oleoylethanolamide (OEA); the fatty acid derivatives, 2-MAG and oleoyl-glycerol (2-OG) (Hansen et al., 2011); the lysophospholipids, lysophosphatidylcholine (LPC) (Soga et al., 2005) and 5 hydroxyl-eicosapentaenoic acid (5-HEPE) (Kogure et al., 2011).

In 2005, Soga *et al.* identified the first GPR119 endogenous agonist, LPC. Ingested material and bile (recently arrived from the gallbladder) generates lysophospholipids,

namely oleic acid, palmitic acid, stearic acid and phosphatidylcholine. Phosphatidylcholine is converted by phospholipase A_2, yielding a potent endogenous GPR119 agonist, LPC in the lumen (Soga et al., 2005). LPC (5μM or 10μM) promoted GSIS in a mouse pancreatic β cell line, NIT-1 transfected with GPR119. This functional effect was partially lost in the presence of GPR119 siRNA. This study suggested LPC was an endogenous GPR119 agonist, which stimulated insulin secretion in mouse NIT-1 cells (Soga et al., 2005).

The ethanolamine derivative, OEA was identified as a lipid mediator that potently reduced food intake and body weight, and additionally enhanced satiety in rats (Rodríguez de Fonseca et al., 2001). These OEA (administered via intraperitoneal injection, i.p.) satiety-inducing effects were apparently PPARα receptor-mediated in mice (Fu et al., 2003). In 2006, OEA was described as a full endogenous GPR119 agonist, which was more potent (EC_{50} 3.2 ± 0.33 μM) than LPC (EC_{50} >30 μM) at the human GPR119 receptor, in a yeast fluorometric assay (Overton et al., 2006). Furthermore, OEA (EC_{50} 2.9 ± 0.46 μM) upregulated cAMP levels in HEK293 cells transfected with human GPR119, and this response was GPR119-mediated (Overton et al., 2006). OEA (30 mg/kg, i.p.) also induced hypophagia in rats, and Overton *et al.* (2006) proposed this may be attributed to GPR119. Later, in 2009, OEA (30 mg/kg, i.p.) suppressed food intake in GPR119 WT and $GPR119^{-/-}$ mice, confirming this anorexigenic effect was GPR119-independent (Lan et al., 2009). OEA is found in small quantities in the diet and is also synthesised on demand from phospholipids within the membrane lipid bilayer, via the N-acyl phosphatidylethanolamide (NAPE) phospholipase D (PLD) pathway. OEA can undergo further enzymatic hydrolysis by the intracellular membrane bound fatty acid amide hydrolase (FAAH), to provide oleic acid and ethanolamine (Cravatt et al., 1996). Lauffer *et al.* (2009) demonstrated that OEA (10 μmol/l) increased basal GLP-1 in murine GLUTag cells (2.1 ± 0.2-fold), which was augmented in the presence of the FAAH inhibitor, URB597 (to 3.2 ± 0.4-fold of control). This functional effect was GPR119-dependent. As a result, OEA-induced GLP-1 release was GPR119-mediated and also limited by FAAH (Lauffer et al., 2009). More recently, Moss *et al.* (2016) showed OEA stimulated GLP-1 release in mouse duodenum/jejunum/colonic primary cultures, and this functional effect was abrogated in $GPR119^{-/-}$ colonic cultures, but not duodenum/jejunum cultures. This suggested OEA induced GLP-1 release was GPR119-mediated in the colon and was

GPR119-independent in the duodenum/jejunum. This indicated regional differences exist in OEA-GPR119 signalling (Moss et al., 2016). 5-HEPE is generated from the hydrolysis of the LCFA, eicosapentaenoic acid by the enzyme 5-hypoxgenase. In 2011, Kogure *et al.* (2011) showed that 5-HEPE and OEA were equipotent activators of GPR119 and suggested 5-HEPE as another endogenous GPR119 agonist. In this study, both OEA and 5-HEPE caused GPR119-dependent insulin release from MIN6 insulinoma cells. While 5-HEPE may be a GPR119 endogenous agonist, it is rarely synthesised unless a large quantity of fish oil is consumed (Kogure et al., 2011).

Trioleoylglycerol digestion by pancreatic lipase yields 2-OG and 2-MAG (e.g. oleic acid) (Hansen et al., 2012). Approximately 5-7 g of 2-OG is yielded per meal in the intestinal lumen. As 2-OG was structurally related to OEA, it was thought to be another potential endogenous GPR119 ligand (Hansen et al., 2012). Initial investigations showed 2-OG (EC_{50} 2.5 μM) amplified cAMP less potently than OEA (EC_{50} 0.2 μM) in COS-7 cells transfected with human GPR119, and both responses were GPR119-mediated. In a human trial, a bolus of 2 g 2-OG (via a duodenal tube) resulted in a rise in plasma GLP-1 and GIP, but not PYY (Hansen et al., 2011). More recently, Hassing *et al.* (2016a) demonstrated that 2-OG mediated GLP-1 release, and this was GPR119-dependent *in vitro* and in *ex vivo* primary colonic cultures (Hassing et al., 2016a). These studies suggest 2-OG may be another endogenous ligand at GPR119, and activation of this receptor causes the release of GLP-1 and GIP in man (Hansen et al., 2011).

N-oleoyldopamine (OLDA) was discovered in the peripheral mesenteric tissue as a lipid derivative of dopamine and was also thought to be a GPR119 endogenous agonist. Chu *et al.* (2010) revealed OLDA was similarly potent to OEA, promoting insulin release in insulinoma cell lines (NIT-1 (mouse), RIN-5F (rat), HIT-T15 (hamster)) expressing human GPR119. Furthermore, OLDA stimulated GIP release and improved glucose-tolerance in mice *in vivo* and these effects were impaired in $GPR119^{-/-}$ mice (Chu et al., 2010). This suggested OLDA was a strong contender as a GPR119 endogenous agonist. Tyrosine hydroxylase and dopacarboxylase were known enzymes responsible for synthesising dopamine from tyrosine, and further N-acylation of newly synthesised dopamine by oleic acid was thought to form OLDA. However, when RNA levels of tyrosine hydroxylase and dopacarboxylase were analysed by RT-PCR, Chu *et al.* (2010) observed low levels in all small intestinal

regions and was unable to detect any RNA in mouse colon, suggesting dopamine/OLDA were not synthesised here via this pathway. Recently, Tough *et al.* (2018b) demonstrated OLDA anti-secretory responses were no different between WT and GPR119$^{-/-}$ mouse descending colon mucosa. This indicated OLDA anti-secretory PYY-mediated responses were GPR119-independent (Tough et al., 2018b). Taken together, these studies demonstrate that OLDA is unlikely to be an endogenous agonist of the GPR119 receptor in the colon.

1.10.2 GPR119 signal transduction in enteroendocrine L cells and pancreatic β cells

Initial studies demonstrated that the potential GPR119 endogenous agonist, LPC elevated GSIS (in 16.8 mM glucose) in an isolated pancreas perfused rat model and a mouse pancreatic β cell line (NIT-1). These responses were inhibited in the presence of the AC inhibitor, MDL12330A implicating LPC-stimulated release was coupled to $G_{\alpha s}$ and was AC/glucose-dependent. In the same study, LPC increased cAMP (EC_{50} 1.5 ± 0.17) in RH777 rat hepatoma cells transfected with GPR119, but not in mock-transfected cells, suggesting the LPC $G_{\alpha s}$ signalling was GPR119-mediated (Soga et al., 2005). Chu *et al.* (2007) were the first group to show GPR119 was constitutively active, a characteristic of many rhodopsin-like GPCRs (Behan and Chalmers, 2001). In their study, a significant rise in cAMP was observed upon expression of the GPR119 receptor in HEK293 cells, compared with cells transiently expressing an empty-vector. This confirmed the GPR119 receptor coupled to $G_{\alpha s}$ and was constitutively active (Chu et al., 2007). Recently, the high constitutive activity of the GPR119 receptor was characterised in COS-7 cells (determined by an ELISA) (Engelstoft et al. 2014). Engelstoft *et al.* (2014) revealed GPR119 constitutive activity was 37 ± 0.8 % of the E_{max} of the cAMP response to GPR119 agonism (AR231453) (Engelstoft et al., 2014).

The two most widely published GPR119 synthetic agonists that have been utilised to interrogate the pharmacology and signalling of the GPR119 receptor, were PSN632406 (Overton et al., 2006) and AR231453 (Semple et al., 2008). In 2006, PSN632408 (developed by Prosidion) was selected during a high throughput screen of small molecules. This small molecule was assessed in a yeast-based assay expressing mouse (EC_{50} 7.9 ± 0.7 μM) or human (EC_{50} 5.6 ± 0.1 μM) GPR119 isoforms. This selective full agonist was as efficacious as OEA and an equipotent

activator of GPR119. PSN632408 increased cAMP (EC_{50} 1.9 ± 0.14 µM) in HEK293 cells transfected with human GPR119. This response was abrogated in cells transfected with an empty vector, and this suggested that activation of the GPR119 receptor leads to $G_{\alpha s}$ signalling (Overton et al., 2006). Similarly, Ning *et al.* (2008) revealed PSN632408 triggered $G_{\alpha s}$-cAMP accumulation in HEK293 cells and GSIS in mouse MIN6c4 insulinoma cells (Ning et al., 2008). Theoretically, as the GPR119 receptor couples to $G_{\alpha s}$, this could result in a rise in cAMP formation, activating PKA and the exchange protein directly activated by cAMP (Epac), to cause release of L cell peptide hormones (Figure 1.12). Notably, PSN632408 has suppressed food intake and reduced body weight and adiposity in rats (Overton et al., 2006). Furthermore, PSN632408 (and OEA) increased the number of β cells in mouse islets *in vitro* and in a transplanted graft in diabetic mice *in vivo,* which improved glycemia after 4 weeks (Gao et al., 2011). This demonstrates GPR119 activation may also be involved in regulating food intake, body weight and β cell protection *in vivo*. The commercially available GPR119 full agonist, PSN632408 was selected as a proven GPR119 full agonist in this PhD study.

AR231453 (developed by Arena Pharmaceuticals) was discovered in 2008 as the first orally potent subnanomolar (≤ nM) agonist for the human and mouse GPR119 receptor. Initial studies revealed AR231453 elevated cAMP (EC_{50} 5.7 ± 1.6 nM) in HEK293 cells transfected with human GPR119. In the same study, AR231453 also triggered GSIS in β cell lines and mouse islets (the glucose concentration ranged from 8-17 mM). This indicated GPR119-mediated insulinotropic effects were glucose-dependent. Chu *et al.* (2008) showed that AR231453 potently induced secretion of GLP-1 in GLUTag cells *in vitro*. This effect was enhanced in the presence of a dipeptidyl peptidase-4 (DPPIV) inhibitor, sitagliptin and abrogated in the presence of a GLP-1R antagonist, exendin (Ex)9-39 (Chu et al., 2008). This confirmed the response was GLP-1-mediated and limited by DPPIV activity *in vivo*. Furthermore, AR231453 also improved glucose tolerance and enhanced GSIS in C57BL/6J (and diabetic) mice, and these effects were eliminated in $GPR119^{-/-}$ mice *in vivo*. Thus, the beneficial effects of this agonist, AR231453 were GPR119-mediated in mice (Chu et al., 2008).

The phenotype of $GPR119^{-/-}$ mice appeared normal, but the GSIS response to a selective GPR119 agonist was impaired (Chu et al., 2008). Similarly, in another study,

GPR119$^{-/-}$ mice were healthy and fertile and no change was observed in behaviour, body weight, leptin, insulin, plasma glucose, morphology of the islets and insulin content (Lan et al., 2009). Thus, whole-body deletion of GPR119 was not detrimental, suggestive of compensatory mechanisms.

Both PSN632408 (Cox et al., 2010) and the more selective GPR119 agonist, PSN-GPR119 (Patel et al., 2014) have elicited anti-secretory responses in mouse and human mucosa. These responses were mediated via a paracrine PYY-Y_1 mechanism and were glucose-sensitive (Cox et al., 2010; Patel et al., 2014). This glucose-sensitivity of GPR119 signalling could provide protection against hypoglycaemia in clinical practise.

1.10.3 Other GPR119 agonists

Compound 16 (EC_{50} 68 nM) (Scott et al., 2014) and compound 42 (EC_{50} 56 nM) (Scott et al., 2012) were identified as potent GPR119 agonists in independent cAMP assays, in HEK293S cells overexpressing mouse GPR119. In an OGTT in C57BL/6J mice, compound 16 and compound 42 both enhanced glucose disposal and induced GLP-1 secretion. These effects were enhanced in the presence of a DPPIV inhibitor, sitagliptin and abrogated in GPR119$^{-/-}$ mice. This study revealed selective GPR119 activation triggered GLP-1 release and this response was limited by DPPIV activity. Compound 16 and 42 were synthesised by AZ and in this thesis, have been abbreviated to Cpd.16 and Cpd.42.

Two GPR119 agonists have progressed into clinical trials, JNJ-28630368 (synthesised by Johnsson and Johnsson Pharmaceutical Research) and GSK1292263 (developed by GSK). However, both clinical investigations were terminated. The JNJ-28630368 clinical trial was terminated due to an unknown reason (Semple et al., 2011), and the GSK1292263 investigations were thought to have terminated due to tachyphylaxis (Nunez et al., 2014). Cornall *et al.* (2013, 2015) revealed that GPR119 agonism in skeletal and cardiac muscle cell lines may be detrimental to fatty acid metabolism and oxidation. This study suggested careful analysis and *in vivo* experiments would be required to investigate the safety of GPR119 agonism in cardiac and skeletal tissue, in order to prevent the failure of future GPR119 clinical compounds (Cornall et al., 2013; Cornall et al., 2015). These findings suggested targeting GPR119 may not be

as safe as originally thought and this may have impeded the progress of many GPR119 projects in the pharmaceutical industry.

There are currently no GPR119 antagonists commercially available.

1.11 Colonic L cell-containing peptides and their physiological significance

1.11.1 Peptide YY and the neuropeptide Y family

PYY, a 36 amino-acid peptide was first isolated in a chemical assay that identified COOH-terminal amides and measured tyrosine-amide release after peptide degradation with trypsin or thermolysin, in porcine small intestine. It was designated PYY, as tyrosine (Y) was present at both terminals (COOH and NH_2 ends) of the amino acid sequence (Tatemoto and Mutt, 1980; Tatemoto, 1982). PYY along with NPY and PP are members of the pancreatic polypeptide family, and all three peptides share a conserved tertiary structure known as the PP hairpin-fold motif. The PP fold is U-shaped with an extended polyproline helix and an α-helix linked to a β-turn (Larhammar, 1996a). PYY, NPY and PP bind NPY receptors denoted as Y receptors, which belong to the rhodopsin class I GPCR family. To date, five distinct mammalian Y receptors have been cloned and characterised, Y_1, Y_2, Y_4, Y_5 and y_6. The Y receptors are GPCRs that couple to the pertussis-toxin sensitive G-protein, $G_{\alpha i}$ and cause inhibition of AC and cAMP elevation (Michel et al., 1998). In mammals, PYY and NPY exhibit the highest affinity for Y_1, Y_2 and Y_5. These three Y receptors possess very low amino acid sequence homology, approximately 27 - 31 % (Larhammar et al., 2001). The Y_4 receptor preferentially binds PP (Bard et al., 1995) and the y_6 receptor is a pseudogene in primates (Matsumoto et al., 1996), but is a functional receptor in mouse (Weinberg et al., 1996) and rabbit (Matsumoto et al., 1996). The existence of a sixth Y receptor subtype, Y_3 was thought to be NPY-preferring, but has not been cloned and therefore will not be mentioned further (Glaum et al., 1997; Lee and Miller, 1998). There are two major circulating forms of PYY including, $PYY_{(1-36)}$ and its truncated form, $PYY_{(3-36)}$ (Eberlein et al., 1989). As $PYY_{(1-36)}$ is released, 40 % is converted to $PYY_{(3-36)}$ (Grandt et al., 1992). $PYY_{(3-36)}$ is the predominant postprandial circulating form (Grandt et al., 1994) and is yielded during degradation of $PYY_{(1-36)}$ by the exopeptidase, DPPIV (Mentlein et al., 1993a) and aminopeptidase-P (Medeiros and Turner, 1994). DPPIV exists in two isoforms, a membrane-anchored form and a

soluble circulating form (Mulvihill and Drucker, 2014). In the GI tract, DPPIV expression has been identified in the epithelium microvilli brush border and capillary endothelium (Hansen et al., 1999). Enzyme degradation of $PYY_{(1-36)}$ to $PYY_{(3-36)}$ does not abolish this peptide's affinity for Y receptors, however the pharmacology of the truncated peptide is altered. The full peptide, $PYY_{(1-36)}$ is selective for Y_1, Y_2 and Y_5. In contrast, $PYY_{(3-36)}$ is more selective as it exhibits a higher affinity for Y_2 and also binds the Y_5 receptor, but with less affinity (Grandt et al., 1992; Grandt et al., 1994).

PYY was localised in 50 % of EECs in the mouse (Arantes and Nogueira, 1997) and human intestinal mucosa (Adrian et al., 1985). The immunoreactivity of PYY increased in frequency along the length of the GI tract, and was found in largest abundance in the terminal ileum, colon and rectum (Adrian et al., 1985; Ekblad and Sundler, 2002). Sparse PYY immunoreactivity was identified in enteric and CNS neurons in comparison to intestinal enteroendocrine PYY immunoreactivity (Ekblad and Sundler, 2002). In enteroendocrine colonic L cells, PYY was co-expressed and co-packaged alongside glicentin (includes OXM), GLP-1 and GLP-2 (Böttcher et al., 1984; Böttcher et al., 1986; Cho et al., 2015). In small intestinal L cells, PYY was co-expressed with mRNA for NTS (Egerod et al., 2012), GIP and CCK (Habib et al., 2012).

Circulating $PYY_{(3-36)}$ rises as early as 15 min after a meal. However, the majority of PYY containing L cells are expressed in the large intestine. This suggests a neuronal signalling mechanism exists, transmitting a signal from the small intestine to the large intestine (Lin et al., 2000), to cause the plasma levels of $PYY_{(3-36)}$ to rise swiftly after a meal. $PYY_{(3-36)}$ peaks again at 90–120 min after a meal, an indicator of macronutrients arriving in the distal regions of the intestine. Notably, the quantity of PYY release is dependent on the size of a meal and the type of macronutrients present in the GI lumen (Adrian et al., 1985).

1.11.2 Y_1 receptor

The Y_1 receptor was the first Y receptor cloned, utilising rat complimentary deoxyribonucleic acid (cDNA) (Eva et al., 1990; Krause et al., 1992). Later, Y_1 receptor clones were also identified in human (Herzog et al., 1992; Larhammar et al., 1992), mouse (Eva et al., 1992) and frog (*Xenopus laevis*) (Blomqvist et al., 1995). The Y_1 receptor sequence is highly conserved between the mouse and rat (98 %) and between rodents and human (94 %) (Larhammar, 1996b). In humans, Y_1 receptor mRNA expression has been identified in the intestine, kidney, blood vessels, ganglia in the ENS plexuses (Wharton et al., 1993) and the brain (cerebral cortex and striatum) (Caberlotto et al., 1997). In the human intestine, the Y_1 receptor was identified in PYY-negative enterocytes (Mannon et al., 1999). Mannon *et al.* (1999) revealed the Y_1 receptor was trafficked to the basolateral membrane, suggesting PYY released from enteroendocrine L cells may act via a paracrine pathway, to activate Y_1 receptors expressed in the surrounding PYY-negative enterocytes. In the mouse, Y_1 mRNA was also found in the intestine and in pancreatic β cells, adipose, heart, kidney, spleen, skeletal muscle, lung and brain (Nakamura et al., 1995).

NPY and PYY were identified as the most potent and efficacious endogenous ligands of the human Y_1 receptor, whereas PP had no affinity at physiological concentrations (Herzog et al., 1992; Larhammar et al., 1992). In mouse (Cox et al., 2001; Tough et al., 2006) and human (Cox and Tough, 2002) colonic mucosa, PYY, NPY and human PP have decreased enterocyte epithelial ion transport, revealing $G_{\alpha i}$-signalling. The PYY anti-secretory response was TTX-insensitive and 90 % inhibited by the Y_1 competitive antagonist, BIBO3304 (Wieland et al., 1998) (IC_{50} 25.4 nM (16.2–39.7 nM), (Cox et al., 2001)). BIBO3304 revealed basal Y_1 tone in human colonic mucosal preparations and inhibited $PYY_{(1\text{-}36)}$, but not human PP or $PYY_{(3\text{-}36)}$ anti-secretory responses (Cox and Tough, 2002). Hence, PYY anti-secretory responses were predominantly Y_1-mediated and epithelial in origin, which was suggestive of a paracrine PYY-Y_1 mechanism, that was also tonically active (Cox et al., 2001; Cox and Tough, 2002; Hyland et al., 2003).

The function of the Y_1 receptor in the GI tract includes PYY-induced inhibition of GI motility (Spiller et al., 1984; Pironi et al., 1993; Lin et al., 1996; Tough et al., 2011). Pedrazzini *et al.* (1998) demonstrated $Y_1^{-/-}$ mice possessed an increased body weight

and a decreased food intake, compared to WT (Pedrazzini et al., 1998). This implicates the involvement of the Y_1 receptor in central mechanisms modulating body weight and food intake (Pedrazzini et al., 1998; Kanatani et al., 2000).

1.11.3 Y_2 receptor

In 1997, the Y_2 receptor was cloned from human SMS-KAN cells and human brain cDNA libraries, while studying the hydrolysed forms of PYY and NPY (Rose et al., 1997). Despite showing only 31 % homology to the Y_1 receptor, the Y_2 receptor was similarly activated by NPY and PYY. However, the truncated forms of NPY and PYY possessed higher affinity for the Y_2 receptor. In contrast, human PP exhibited the lowest potency for the Y_2 receptor (Michel et al., 1998; Cox et al., 2001; Cox and Tough, 2002; Hyland et al., 2003). Like Y_1 signalling, activation of the Y_2 receptor led to inhibition of AC and cAMP accumulation (Michel et al., 1998). Y_2 receptor expression has been identified mainly in brain pre-synaptic neurons, where it appears to be an autoreceptor (Colmers et al., 1991), and was rarely observed in the periphery (Gehlert et al., 1996). In human colonic mucosa, the Y_2 antagonist, BIIE0246 (Doods et al., 1999) revealed Y_2 tone and inhibited $PYY_{(3\text{-}36)}$, not human PP or $PYY_{(1\text{-}36)}$ anti-secretory responses. Furthermore, $PYY_{(3\text{-}36)}$ anti-secretory responses were tetrodotoxin (TTX)-sensitive. Together, this data indicated that $PYY_{(3\text{-}36)}$ responses were neuronally-mediated and not epithelial in origin (Cox and Tough, 2002). A year later, Hyland *et al.* (2003) demonstrated that $PYY_{(3\text{-}36)}$ displayed an EC_{50} of 10.1 nM (male) and 10.2 nM (female) in $Y_2^{+/+}$ mouse colonic mucosa and these anti-secretory responses were lost in $Y_2^{-/-}$ mucosa. Additionally, the Y_2 antagonist (BIIE2046) revealed Y_2 tone, which was also lost in $Y_2^{-/-}$ mucosa, revealing tonic endogenous Y_2 activity. Hyland *et al.* (2003) also revealed the Y_2 receptor was prejunctional in mucosa and post-junctional in longitudinal smooth muscle. Centrally, $PYY_{(3\text{-}36)}$ is a major anorexigenic mediator and its effects appear to be mediated by the Y_2 receptor (Batterham et al., 2002; Batterham et al., 2003; Abbott et al., 2005). These mechanisms will be introduced in section 1.14. The phenotype of $Y_2^{-/-}$ mice was lean and showed a reduction in body weight gain (larger reduction in females), adiposity, elevated PP levels and an increased food intake, implicating the involvement of the Y_2 receptor in appetite and body weight modulation (Sainsbury et al., 2002).

1.11.4 Y_4 receptor

In 1995, the Y_4 receptor was cloned utilising the human genomic library (Lundell et al., 1995) and was closely related to the Y_1 receptor (42 % amino-acid sequence homology) (Darby et al., 1997). In humans, this receptor was localised in the small intestine and colonic mucosa, stomach, pancreas and prostrate (Bard et al., 1995). In the mouse, Y_4 was observed in the heart, intestine and brain (Michel *et al*., 1998). In 1975, PP was isolated from avian pancreas, released from pancreatic endocrine F cells (approximately 10 % of pancreatic islets) (Kimmel et al., 1975). PP possessed the highest affinity for Y_4, followed by PYY and NPY for the human Y_4 receptor in COS-7 cells (Bard et al., 1995). PP was observed relatively rarely in the mouse or human small intestine/colon and was not co-localised with PYY (Ekblad and Sundler, 2002). In human colonic mucosa, Y_4 receptors were expressed in epithelia as Y_4 agonism was TTX-insensitive (Cox and Tough, 2002). In 2006, Tough *et al.* established rat PP induced anti-secretory responses resembling $G_{\alpha i}$-signalling in mouse colonic mucosa, and these responses were abolished in $Y_4^{-/-}$ mice, further demonstrating a role for Y_4 in colonic epithelial ion transport (Tough et al., 2006). The phenotype of $Y_4^{-/-}$ mice show a reduced food intake and body weight gain, thus validating the involvement of Y_4 receptors in feeding (Sainsbury et al., 2010).

1.11.5 Y_5 receptor

In 1996, Y_5 was cloned from a hypothalamic rat cDNA library (Gerald et al., 1996; Hu et al., 1996) and possessed 30 - 33 % amino acid sequence homology to Y_1, Y_2 and Y_4 (Hu et al., 1996). Initial studies revealed that the rat Y_5 receptor couples to $G_{\alpha i}$ in HEK293 cells (Gerald et al., 1996). In two independent studies, NPY and PYY activated the Y_5 receptor with similar potencies, whereas PP exhibited the lowest potency (Gerald et al., 1996; Hu et al., 1996). Y_5 was predominately expressed in the CNS, where it strongly regulated appetite and was observed sparsely in the periphery (Gerald et al., 1996). In humans, Y_5 expression has not been observed in the small intestine or colon (Michel et al., 1998). Furthermore, the Y_5 agonist ([Ala31, Aib32]NPY) (Cabrele et al., 2000) had no effect on epithelial ion transport in human mucosa (Cox and Tough, 2002). Moreover, this Y_5 agonist was only responsive at micromolar concentrations (μM) in mouse descending colon mucosa (Cox et al.,

2001). These studies suggested Y_5 is not involved in electrolyte transport in human intestine and appears to have no role in mouse mucosa. $Y_5^{-/-}$ mice remain healthy but develop late onset obesity around 30 + weeks, and this was attributed to increased food intake and body weight gain (Kushi et al., 1998). More recently, Ishihara and colleagues examined the effects of a selective Y_5 antagonist in mice on a HFD. These mice displayed a reduction in fat pad weight, insulin-levels and an inhibition of diet-induced body weight gain, compared to untreated mice (Ishihara et al., 2006). These studies implicate the Y_5 receptor as a critical regulator of appetite, satiety and body weight.

1.11.6 y_6 receptor

The y_6 receptor was initially cloned from murine genomic DNA (Weinberg et al., 1996). Later, in man (and primates) this receptor was identified as a pseudogene attributed to a frameshift mutation (single-base mutant) in the third intracellular loop, which encoded a non-functional truncated protein (Matsumoto et al., 1996; Michel et al., 1998). As a result, this receptor was designated a lower-case y. Despite the non-functionality of this receptor (y_6) in man, it shares 50 % sequence homology with Y_1 and Y_4 (Wraith et al., 2000). In humans, y_6 has been identified in the small intestine, colon, skeletal muscle and cardiac muscle (Matsumoto et al., 1996). The murine y_6 receptor was functional and localised in the small intestine and was sparsely expressed in embryonic colon and brain (Weinberg et al., 1996). The agonist potency rank at the murine y_6 receptor has been controversial between studies. One study showed a rank of PP > PYY ≥ NPY (similar to Y_4 receptor) (Gregor et al., 1996) whereas, another study displayed the opposite order NPY = PYY > PP (similar to the Y_1 receptor) (Weinberg et al., 1996). The potency rank was reinvestigated in HEK293 cells expressing mouse y_6. Mullins *et al.* (2000) revealed PYY and NPY exhibited equipotent binding affinities at the mouse y_6 receptor in HEK293 cells. Both agonists inhibited cAMP accumulation showing $G_{\alpha i}$-signalling (Mullins et al., 2000) and thus, their potency rank was similar to that observed in an earlier study (Weinberg et al., 1996). Taken together, while y_6 appears to be functional in the mouse, the pharmacology of this receptor has not been well defined. Furthermore, Mullins *et al.* (2000) did not suggest a role for y_6 in feeding or appetite regulation.

1.12 Glucagon-like peptides

GLP-1 and GLP-2 are co-encoded within the proglucagon gene. Proglucagon undergoes differential tissue-specific post-translational processing, in pancreatic α cells and GI enteroendocrine L cells, yielding various bioactive products (Baggio et al., 2004) (Figure 1.14). In the L cell, proconvertase (PC)-1/3 cleaves the proglucagon gene to form GLP-1, GLP-2, intervening peptide-2 (IP-2) and glicentin. Glicentin is cleaved further creating glicentin-related pancreatic polypeptide (GRPP) and OXM. In pancreatic α cells, PC-2 yields glucagon, intervening peptide-1 (IP-1) and GRPP (Holst, 2007). Like PYY, the quantity of GLP-1/GLP-2 release is dependent on the calorie intake amount and the type of macronutrients. The GLP-1/GLP-2 peptides are secreted in a 1:1 ratio (Lim and Brubaker, 2006). On average, plasma levels of GLP-1/GLP-2 increase 2-5-fold after a meal (Xiao et al., 1999; Nauck et al., 2011). The L cell postprandial GLP secretion is biphasic. The first phase is rapid, peaking within 15-30 min and has been attributed to neuronal signals arriving from the proximal intestine. The second phase of GLP-1 plasma levels peak 90-120 min later and is attributed to direct stimulation of L cells by dietary macronutrients (Brubaker, 2006).

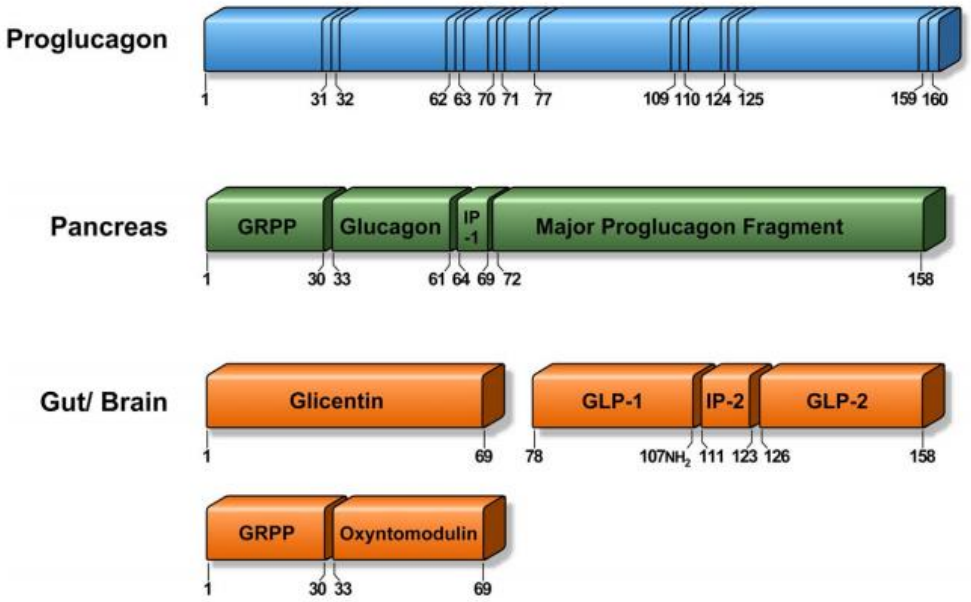


Figure 1.14: Tissue-specific differential posttranslational processing of proglucagon in the pancreas, gut and brain. The numbers indicate amino acid positions in the proglucagon sequence (total 160 amino acids). The vertical lines illustrate specific cleavage sites. GRPP, glicentin-related pancreatic polypeptide; IP-1, intervening peptide-1; IP-2, intervening peptide-2 (Holst, 2007).

1.12.1 Glucagon-like peptide 1

GLP-1 is a member of the incretin glucoregulatory family and constitutes a 36 amino-acid peptide sequence, that is highly conserved among mammals (Kreymann et al., 1987; Mojsov et al., 1987). The two discovered circulating forms of human GLP-1 were GLP-$1_{(7\text{-}37)}$ and GLP-$1_{(7\text{-}36)}$ amide (Ørskov et al., 1994). Once released, these circulating forms were rapidly degraded (cleaving two NH_2-terminal amino acids) within 1-2 min by DPPIV, yielding inactive GLP-$1_{(9\text{-}36)}$ amide and GLP-$1_{(9\text{-}37)}$ in human (Mentlein et al., 1993b). The truncated GLP-$1_{(9\text{-}36)}$ amide was the major circulating form of GLP-1 (Deacon et al., 1995). Hansen *et al.* (1999, 2000) revealed that less than 25 % of active GLP-1 leaves porcine ileum *in vitro* (Hansen et al., 1999; Hansen et al., 2000). In the liver, active GLP-1 can be further degraded (about 40-50 %) before entering the systemic circulation (Deacon et al., 1995). Therefore,

approximately 10 – 15 % of released active GLP-1 may be measured in the circulation. Similarly, in human intestine, 80 % of active GLP-1 was amidated ($GLP\text{-}1_{(7\text{-}36)}$ amide) as it entered the circulation (Ørskov et al., 1994).

GLP-1 is an incretin i.e. a GI hormone released in response to orally ingested macronutrients and subsequently amplifies insulin secretion. Postprandial GLP-1 release was a powerful stimulant of pancreatic β cell insulin secretion (Fehmann and Habener, 1992). Additionally, GLP-1 protected the β cell by enhancing β cell mass, proliferation, inhibiting apoptosis and decreasing α cell mass (Farilla et al., 2002). Hence, GLP-1 was a highly relevant peptide of interest in anti-diabetic therapeutics. Unfortunately, the rapid half-life of GLP-1 limited the therapeutic value of this peptide. As a result, successful GLP-1 mimetics were synthesised to recapitulate the physiological effects of the endogenous GLP-1 peptide. Clinically proven GLP-1 mimetics, like Exenatide (Kendall et al., 2005) or the longer acting, Liraglutide (Zinman et al., 2009) have revolutionised the diabetic therapeutic field, and are the most efficacious drugs currently on the market to treat T2DM. These GLP-1 mimetics improve glycaemic control and promote weight loss (Dushay et al., 2012; Mehta et al., 2017). DPPIV inhibitors e.g. sitagliptin are often given as adjunctive T2DM therapeutics to prevent endogenous peptide degradation (i.e. GLP-1 and PYY), with the added benefit of partial weight loss (Drucker and Nauck, 2006)

In the GI tract, GLP-1 slowed GI motility via GLP-1R (Amato et al., 2010; Halim et al., 2018), may be involved in the ileal brake (Giralt and Vergara, 1999), decreased gastric acid (and pancreatic) secretion (Imeryüz et al., 1997) and slowed gastric emptying (Nauck et al., 2011). Together, these GLP-1-mediated GI effects may enhance central satiety. Indeed, studies have shown GLP-1 decreases food and fluid intake in rats (Tang-Christensen et al., 1996) and humans (healthy and obese) (Flint et al., 1998; Näslund et al., 1998; Gutzwiller et al., 2004). Furthermore, in healthy individuals, GLP-1 enhanced gastric accommodation, which correlated with a significant decrease in the feeling of hunger (Delgado-Aros et al., 2002; Andrews et al., 2006). The anorectic effect of GLP-1 appeared to be mediated by vagal and brainstem feeding pathways in the rat, as this anorectic effect was absent following vagotomy (Imeryüz et al., 1997). Therefore, GLP-1 has anorexigenic properties and most likely contributes to the augmentation of satiety in animal models and humans.

*1.12.2 GLP-1 recepto*r

In 1992, the GLP-1R was cloned using cDNA from rat pancreatic islets (Thorens, 1992), and this was followed by the cloning of the homologous human GLP-1R (Thorens et al., 1993). GLP-1R is a class 2 GPCR, joining the glucagon, GIP and secretin receptor family (Mayo, 2003). Activation of GLP-1R (with $GLP\text{-}1_{(7\text{-}37)}$) resulted in the stimulation of the $G_{\alpha s}$-stimulatory cAMP pathway and the elevation of $[Ca^{2+}]_i$, in COS-7 cells expressing rat GLP-1R (Wheeler et al., 1993). The expression of mouse (Campos et al., 1994) and human GLP-1R (Wei and Mojsov, 1995) has been identified in pancreatic islets, stomach, intestine, heart, kidney and the brain hypothalamus. In 1991, Ex4 was isolated from *Heloderma suspectum* (Glia monster) venom, and it displayed structural similarity (53 %) to mammalian GLP-1. Initial studies demonstrated that Ex4 exhibited $^{125}IGLP\text{-}1_{(7\text{-}36)}$ displacement at the GLP-1R. This indicated Ex4, like GLP-1, selectively activated the GLP-1R. In a separate study, Ex4 triggered cAMP accumulation in rat insulinoma RINm5F cells, suggestive of $G_{\alpha s}$ signalling. Ex4 also increased GSIS in rat islets and a mouse insulinoma cell line, and these effects were suppressed by the truncated form of the Ex4, also known as Ex(9-39) amide (Göke et al., 1993). This study demonstrated Ex4 mimicked the action of GLP-1 and was thus a GLP-1R agonist. Furthermore, Ex(9-39) amide was a selective GLP-1R antagonist. The discovery of Ex4 pioneered the field and led the way forward in the creation of GLP-1 mimetics. Ex4 and Ex(9-39) were key pharmacological tools used in this thesis.

Ablation of the GLP-1R in mice has demonstrated the importance of this receptor in glucose tolerance. $GLP\text{-}1R^{-/-}$ mice present with mild fasting hyperglycaemia and glucose intolerance, which have been attributed to a reduction in postprandial insulin-release and abnormal glucose excursions. These mice also display normal body weight and feeding regulation (Scrocchi et al., 1996), and thus reveal alternative compensatory mechanisms to control feeding and appetite.

1.12.3 Glucagon-like peptide 2

In 1996, glucagon-like peptide 2 (GLP-2) was isolated from mammalian proglucagon DNA, as a 33-amino acid intestinotrophic peptide. In this initial study, Drucker *et al.* (1996) demonstrated that mice treated with GLP-2 for 4-6 days presented with a 50 %

increase in small bowel weight, enhanced crypt cell proliferation and an increase in mucosal thickness in the proximal small intestine. The increase in mucosal thickness was attributed to a narrowing of intestinal cells, elongated microvilli and an inhibition of intestinal crypt enterocyte apoptosis (Drucker et al., 1996). Drucker *et al.* (1997) investigated the intestinotrophic effects of GLP-2 in rodents. Here, GLP-2 stimulated elongation of intestinal villus height in mice and rats. Further investigations revealed GLP-2 was unable to increase intestinal villus height in $DPPIV^{-/-}$ mice, demonstrating DPPIV limits the intestinotrophic effects of GLP-2 (Drucker et al., 1997). Notably, compared to GLP-1, GLP-2 was less susceptible to degradation by the DPPIV enzyme, in both the GI tract and vasculature (Drucker et al., 1997). In humans, $GLP\text{-}2_{(1\text{-}33)}$ is hydrolysed into $GLP\text{-}2_{(3\text{-}33)}$ within 7.2 mins. In this way, most of the released GLP-2 is still intact and efficacious by the time it reaches the circulation (Hartmann et al., 2000).

Unlike GLP-1, GLP-2 is not an incretin. The main physiological functions of GLP-2 are maintenance and protection of the intestinal integrity and morphology (Brubaker et al., 1997; Scott et al., 1998), inhibition of crypt cell apoptosis (Boushey et al., 2001), increasing crypt cell proliferation (Drucker et al., 1996; Drucker et al., 1997) and reducing GI epithelium inflammatory states (Drucker et al., 1999; Sigalet et al., 2007). This includes a reduction in paracellular epithelial tight-junction permeability, which strengthens epithelium barrier function (Benjamin et al., 2000). The activity of epithelium disaccharidases (maltase, sucrase, lactase) was enhanced by GLP-2 treatment (10 days) in mice. This increased activity of disaccharidases may facilitate and enhance the absorption of disaccharides (Brubaker et al., 1997). Certainly, the absorption of the amino acid, leucine and the triglyceride, triolein was increased (Brubaker et al., 1997). More recently, GLP-2 enhanced leucine absorption in mice *in vivo* (Lee et al., 2017), and this implicated the involvement of GLP-2 in the facilitation of nutrient absorption. Having established the effects of GLP-2 in rodent models, investigations were extended to human trials. In humans with short-bowel syndrome (subjects present with no ileum (or colon) and malabsorption), GLP-2 (administered twice daily, subcutaneous injection (s.c) for 35 days) provided beneficial intestinotrophic effects, slowed gastric emptying and improved the nutritional state of these individuals (Jeppesen et al., 2001). The effect of GLP-2 administration on gastric motility has been assessed in mouse and humans. In WT

mice, GLP-2 (0.15 µg/g body weight) slowed upper intestinal transit (by 29 %) in response to a charcoal meal (oral gavage), but was less potent than GLP-1 (55 %) *in vivo* (McDonagh et al., 2007). In humans, the effects on motility have been controversial. In healthy volunteers, Schmidt *et al.* (2003) observed no effect of GLP-2 (0.75 and 2.25 pmol kg body wt $^{-1}$ min $^{-1}$) on the motility of a solid meal (omelette) and suggested GLP-2 had no role to play in the ileal brake. In another study GLP-2 administration (0.5 and 1.0 pmol kg body wt $^{-1}$ min $^{-1}$) resulted in a dose-dependent slowing of antral emptying to a liquid meal (lukewarm beef tea) in healthy volunteers (Nagell et al., 2004) as previously observed (Jeppesen et al., 2001). While it appears GLP-2 slows GI motility in mice, the motility effect in humans remains unresolved. The main beneficial effects of GLP-2 in rodents and humans appears to be intestinotrophic and mucosal protective.

1.12.4 GLP-2 receptor

The glucagon-like peptide 2 receptor (GLP-2R) was cloned from rat intestinal and hypothalamic cDNA libraries. The rat GLP-2R clone was utilised to identify the human GLP-2R (Munroe et al., 1999). Like the GLP-1R, the GLP-2R belongs to the 7-transmembrane class 2 GPCR family (Mayo, 2003). Activation of GLP-2R amplified AC activity and increased cAMP (EC_{50} 0.58 nM) signalling (stimulatory $G_{\alpha s}$ pathways), in COS-7 cells expressing rat GLP-2R (Munroe et al., 1999). Human GLP-2R was expressed in the stomach, intestine and brain (Yusta et al., 2000). In the mouse, GLP-2R was identified in the stomach, duodenum, jejunum, colon, hypothalamus, brain stem and lung (Yusta et al., 2000). More specifically, GLP-2R has been localised in subepithelial myofibroblasts (Ørskov et al., 2005), in enteric neurons, vagal afferents (Guan et al., 2006), in intestinal PYY- and GLP-1-containing EECs (Yusta et al., 2000), the hypothalamus, hippocampus and the nucleus tractus solitarius (Lovshin et al., 2001).

The GLP-2R$^{-/-}$ mice possess normal glucose regulation in various ranges of glycemia and no clear phenotype (Bahrami et al., 2010). However, a GLP-2R deficiency in diabetic ob/ob mice revealed an amplification of α cell mass, a diminished β cell mass, hyperglycaemia, elevated glucagon secretion and glucose intolerance. This

demonstrated that the adaptive trophic features associated with GLP-2R activation were critical in metabolic stress (Bahrami et al., 2010).

1.13 Oxyntomodulin

In the early 1980's OXM (previously known as enteroglucagon) was isolated and characterised from porcine jejuno-ileum (Bataille et al., 1982), as a 37 amino-acid peptide that resembled glucagon. OXM was also isolated in rat (Bataille et al., 1981; Collie et al., 1994) and human intestine (Ghatei et al., 1983). This peptide was produced in preproglucagon-containing cells and was processed similarly to GLP-1 and GLP-2 (Mojsov et al., 1986). The preproglucagon precursor is cleaved by PC 1/3, creating OXM, a peptide formed of glucagon and an octapeptide extension, also known as IP-1 (Figure 1.14) (Holst, 1997). OXM is co-released with GLP-1, GLP-2 and PYY from enteroendocrine L cells (Böttcher et al., 1984; Böttcher et al., 1986). Two independent studies revealed OXM exhibited affinity for the GLP-1R (Gros et al., 1993) and the porcine glucagon receptor (GCGR) (Baldissera et al., 1988), implicating OXM as a dual agonist. At each receptor, OXM was 10 - 100 fold less potent than the cognate ligands, GLP-1 and glucagon (Baldissera et al., 1988; Gros et al., 1993). Despite OXM engaging the same receptor as GLP-1, peripheral administration of OXM and GLP-1 has resulted in differential region-specific hypothalamic neuronal activity (Chaudhri et al., 2006). Utilising manganese-enhanced magnetic resonance imaging, Chaudhri *et al.* (2006) revealed that OXM reduced neuronal activity in the arcuate nucleus (ARC), paraventricular nucleus (PVN) and supraoptic nuclei. In contrast, GLP-1 suppressed neuronal signalling in the PVN and enhanced signalling in the ventromedial hypothalamus (VMH). This suggested that while OXM and GLP-1 both bind the GLP-1R, an uncharacterised OXM receptor may exist. In this way, the observed differences in hypothalamic neuronal signalling (Chaudhri et al., 2006) may be attributed to the activation of this uncharacterised OXM receptor. To date, no specific OXM receptor has been identified or sequenced.

OXM appears to be an anorectic peptide in rodents and humans. Intracerebroventricular (icv) administration of OXM into the PVN suppressed food intake in rats (Dakin et al., 2001; Dakin et al., 2002). This anorectic effect was reduced

in the presence of the GLP-1 antagonist, Ex(9-39), suggestive of a GLP-1R-mediated mechanism (Dakin et al., 2001). In another study, OXM (icv) transiently inhibited food intake, and this effect was absent in GLP-1R$^{-/-}$, not GCGR$^{-/-}$ mice. This confirmed the OXM-induced anorectic effect was GLP-1R-dependent (Baggio et al., 2004). This anorectic effect was also displayed in healthy subjects (Cohen et al., 2003). OXM (i.p.) decreased food intake and suppressed the levels of the appetite stimulant peptide, ghrelin. These anorectic effects of OXM extended to obese and overweight volunteers (Cohen et al., 2003). In another study, self-administration of OXM (s.c, 3 times daily) resulted in a suppressed energy intake, enhancement in activity-induced energy expenditure and lower body weight in overweight and obese volunteers (Wynne et al., 2006). More recently, the reduction in body weight was also shown to be mediated by the GCGR receptor in mice (Kosinski et al., 2012). Together, these studies demonstrated that combined agonism of GLP-1R and GCGR with OXM induced beneficial anorectic effects. OXM also increased insulin secretion from murine islets *in vitro,* improved glucose tolerance and enhanced glucose disposal in WT (Maida et al., 2008) and DIO mice *in vivo* (Parlevliet et al., 2008). These effects were GLP-1R-mediated (Maida et al., 2008; Parlevliet et al., 2008). Other benefits of OXM agonism include protection of β cell mass apoptosis in mice (Maida et al., 2008), reduction of gastric acid/pancreatic exocrine secretions and inhibition of gastric emptying in humans (Schjoldager et al., 1989). Furthermore, OXM had no effect on gastric motility in mice (Maida et al., 2008).

1.14 Control of appetite in the hypothalamic arcuate nucleus and the role of PYY$_{(3-36)}$

The hypothalamus, located in the mediobasal section of the brain and the brainstem are the main centres of energy and appetite regulation (Murphy and Bloom, 2006). The basal region of the hypothalamus forms the ARC. The ARC encloses the third ventricle and is a modified region of the blood-brain barrier (semi-permeable) and is anatomically above the median eminence. This region of the hypothalamus serves as a privileged first sensor of the peripheral energy status of the whole organism, allows entry of peripheral peptides and critically integrates neuronal and endocrine signals (Peruzzo et al., 2000). The ARC consists of two populations of nuclei that either enhance appetite (orexigenic) or inhibit (anorexigenic) food intake (Figure 1.15)

(Cone, 2005). ARC neurons project onto second order neurons in higher hypothalamic regions, the VMH, the paraventricular hypothalamus (PVH) and the lateral hypothalamus area (LHA), to regulate satiety, appetite and energy expenditure (Schwartz et al., 2000).

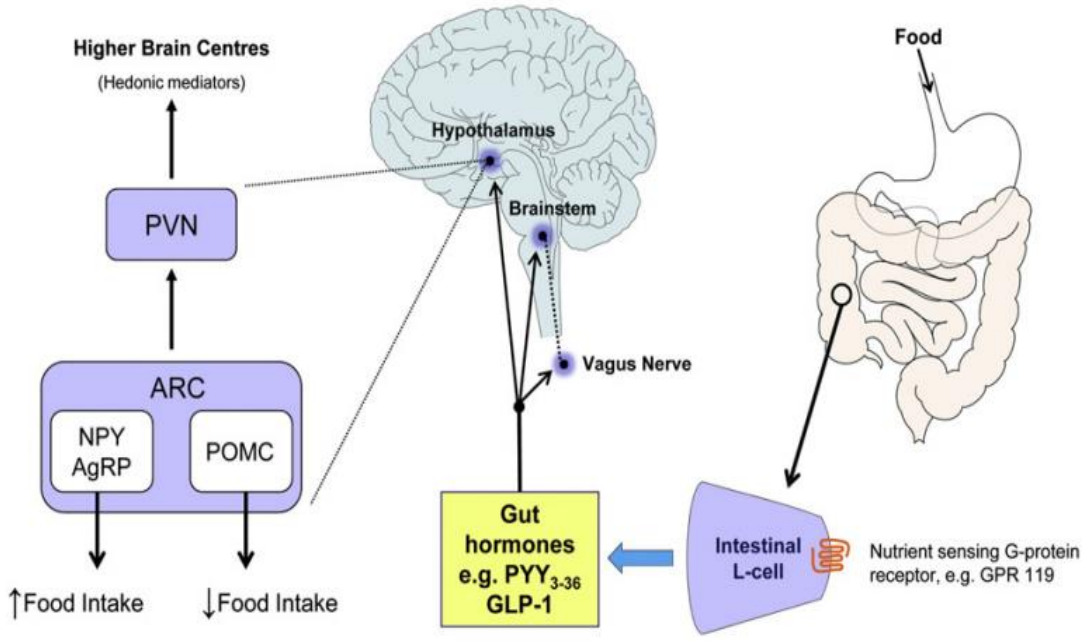


Figure 1.15: Two populations of first order neurons regulate feeding and appetite in the hypothalamic arcuate nucleus (ARC). Stimulated proopiomelanocortin (POMC) neurons in the ARC, release α melanocyte-stimulating hormone (α-MSH). α-MSH activates MC_3/MC_4 receptors expressed on second-order neurons, in the paraventricular nucleus (PVN). This enhances satiety by reducing food intake and stimulating energy expenditure. The second population of neurons in the ARC, are agouti-related peptide (AgRP)/NPY neurons. When stimulated, AgRP, an endogenous antagonist of the MC_4 (and MC_3) receptor, is released and blocks the effects of α-MSH, to increase food intake and reduce energy expenditure. Ingested macronutrients stimulate the release of GI peptides e.g. PYY and GLP-1 from enteroendocrine L cells. These hormones activate hypothalamic nuclei and the brain stem vagal afferents, to regulate feeding and satiety. Therefore, the ARC integrates

neuronal and endocrine signals from the periphery and brainstem. Image from Sam *et al.* (2012).

The lateral located anorexigenic neurons in the ARC, coexpress cocaine and amphetamine-related transcript (CART) (Koylu et al., 1997; Kristensen et al., 1998) and proopiomelanocortin (POMC) neurons (Elias et al., 1998). These neurons are powerful suppressants of food intake and body weight. POMC is cleaved by PC 1/2 within these neurons, yielding α melanocyte-stimulating hormone (α-MSH) (Jacobowitz and O'Donohue, 1978; Cone et al., 1996). α-MSH binds central G-protein coupled melanocortin receptors (MC_3 (Roselli-Rehfuss et al., 1993) and MC_4 (Cone et al., 1996)), to inhibit feeding and enhance energy expenditure (Fan et al., 1997). Notably, MC_4 is highly expressed in the hypothalamic PVN (Mountjoy et al., 1994). Target deletion of MC_4 results in hyperphagia and obesity in mice (Huszar et al., 1997) and humans (Vaisse et al., 1998; Yeo et al., 1998). Conversely, the medially located orexigenic neurons that coexpress AgRP (Shutter et al., 1997; Haskell-Luevano et al., 1999) and NPY (Broberger et al., 1998) stimulate food intake. AgRP is a potent endogenous MC_3 and MC_4 competitive antagonist (Lu et al., 1994; Ollmann et al., 1997) that inhibits the effect of α-MSH, which stimulates feeding and decreases energy expenditure (Broberger et al., 1998; Hahn et al., 1998; Hagan et al., 2000). The regulation and balance between the orexigenic and anorexigenic neuronal populations ensures food intake and energy expenditure is maintained.

Ingested macronutrients stimulate the release of numerous GI peptides. Initial studies suggested acute peripheral $PYY_{(3-36)}$ administration reduced food intake in rodents (Batterham et al., 2002) and humans (healthy and obese) (Batterham et al., 2003). However, these studies were controversial as many laboratories were unable to repeat these findings. The inconsistencies between studies have been attributed to animal stress, and the importance of prior acclimatisation (i.e. animal handling) of each experimental animal to the methodology and environment (Tschöp et al., 2004). Batterham *et al.* (2002) demonstrated the reduction in food intake was mediated via the ARC, as peripherally administered $PYY_{(3-36)}$ increased c-fos expression in this region. In this study, $PYY_{(3-36)}$ hyperpolarised non-POMC neurons (which were assumed as orexigenic NPY neurons) and this caused disinhibition, and thus depolarisation of anorexigenic POMC neurons. The anorectic effect of $PYY_{(3-36)}$ was absent in $Y_2^{-/-}$ mice, indicating this $PYY_{(3-36)}$-induced anorectic effect was Y_2-

mediated (Batterham et al., 2002). Subsequent studies, have confirmed $PYY_{(3-36)}$ elicited an acute reduction in food intake in mice (Challis et al., 2003; Pittner et al., 2004; Adams et al., 2006), rats (Abbott et al., 2005; Chelikani et al., 2006) and rhesus monkeys (Moran et al., 2005) *in vivo*. Abbott *et al.* (2005) demonstrated food intake was inhibited in mice treated with the Y_2R antagonist, BIIE2046 *in vivo* (Abbott et al., 2005). Taken together, these studies show that inhibition of food intake by $PYY_{(3-36)}$ was Y_2-mediated, which resulted in the suppression of ARC orexigenic NPY neurons and an increased neuronal activity of anorexigenic POMC neurons.

1.15 PYY slows the colonic and ileal brakes

Initial intestinal motility investigations have described the slowing of small bowel transit to an ileal perfusion of a lipid emulsion in human subjects (Read et al., 1984; Spiller et al., 1984; Spiller et al., 1988). These studies revealed the existence of an inhibitory intestino-intestinal feedback mechanism, whereby unabsorbed nutrients in the ileum delayed proximal intestinal motility (Read et al., 1984). This mechanism, designated the ileal brake (Spiller et al., 1988) was critical to slow the motility of luminal macronutrients, to optimise digestive and absorptive processes. The companion brakes include, the duodenal brake, jejunal brake and the colonic brake (Van Citters and Lin, 1999). PYY was a major inhibitory mediator of intestinal motility, as studies showed a positive correlation between PYY release and reduced intestinal motility following a lipid stimulation in humans (Pironi et al., 1993). The use of a polyclonal PYY antibody abolished the PYY-induced inhibition of intestinal transit, confirming PYY was the primary mediator of the ileal brake (Lin et al., 1996). In another study, PYY also slowed colonic motility in cats (Lundberg et al., 1982). The ability of distal released PYY to inhibit proximal intestinal motility most likely involves activation of the intestinofugal nerve pathway, which passes through the sympathetic prevertebral ganglia (Figure 1.8) (Furness et al., 2013). Lin *et al.* (2000) showed that distal PYY release was dependent on proximal CCK release. This suggested that newly arrived chyme in the small intestine stimulated upper intestinal I cells to release CCK, and this in turn signalled distal L cells to secrete PYY (Lin et al., 2000). In this way, PYY initiated the ileal brake (and the associated compound brakes), which slowed small intestinal motility to allow optimal absorption of

nutrients. Furthermore, serotonergic, opioid (Lin, 2004) and β-adrenergic pathways (Lin et al., 2003) were also thought to contribute to the inhibition of intestinal transit by PYY in canines.

While PYY is a critical regulator of GI motility, normal peristalsis also requires functional circular and longitudinal smooth muscle, the ENS/CNS innervation, and ICCs to generate peristaltic slow waves (Bornstein et al., 2004). Dysfunction in any of these three components also result in abnormal or defective GI motility. Indeed, the importance of the ENS is observed in Hirschsprung disease. Infants with this disorder lack ENS development in the distal intestine, and therefore this region of the intestine is unable to propel chyme appropriately (Furness, 2012). Prolonged and abnormal peristalsis is also observed in mice with an impaired ICC intestinal network (Ward et al., 1994; Der–Silaphet et al., 1998) and in mice lacking the contractile activity of intestinal smooth muscle cells (Angstenberger et al., 2007). Taken together, these studies reveal the importance of the ENS, ICCs, smooth muscle and PYY to modulate intestinal motility and optimise nutrient absorption.

1.16 Intestinal epithelial ion transport

1.16.1 Intestinal epithelial ion absorption

In humans, 1.3-1.8 L electrolyte-rich fluid is absorbed daily, with 90 % of this occurring in the proximal intestine (Debongnie and Phillips, 1978). The intestinal epithelium consists of predominantly absorptive/secretory enterocytes (Figure 1.16). Enterocytes in the intestinal crypt region are highly secretory compared to surface epithelial enterocytes, that are primarily absorptive (Köckerling et al., 1993; Hermiston and Gordon, 1995). Glucose and amino acids are actively absorbed and co-transported into enterocytes with Na^+. Once in the cell, these nutrients exit the serosal side, independent of ion movement (Field, 2003). The predominate apical absorption of Na^+ and Cl^- in the intestine is electroneutral (via Na^+/H^+ (NHE) and Cl^-/HCO_3^- exchangers) (Rajendran and Binder, 1990; Rajendran et al., 1995) and electrogenic (via apical amiloride-sensitive ENac channels) (Kunzelmann and Mall, 2002). In humans, the proximal colon absorption is mainly electroneutral, and absorption in the distal colon is primarily electrogenic (Levitan et al., 1962).

In the colonic enterocytes, there are three types of NHEs, NHE1, which is located in the serosal membrane, whereas NHE2 and NHE3 are in the luminal epithelial membrane (Figure 1.16). Most electroneutral Na^+ absorption occurs via NHE3 (Ikuma et al., 1999). Upregulation of cAMP inhibits electroneutral NaCl reabsorption, an effect that was absent in mice lacking the predominant luminal Cl^- channel, also known as cystic fibrosis transmembrane conductance regulator (CFTR). This implicates a regulatory role of CFTR in electroneutral NaCl absorption in the intestine (Clarke and Harline, 1996). Electroneutral absorption of Cl^- occurs via surface epithelium luminal Cl^-/HCO_3^- or Cl^-/OH^- exchangers (also present in crypt cells). Furthermore, a third Cl^-/HCO_3^- exchanger is located in the serosal membrane, providing a passage for Cl^- to enter the circulation (Rajendran et al., 2000). As Cl^- exits, HCO_3^- enters the cytosol and is secreted via the luminal Cl^-/HCO_3^- exchanger, creating an alkaline intestinal region (Seidler et al., 2000).

Electrogenic transport of Na^+ occurs via luminal ENac channels and this generates an electrochemical gradient (Canessa et al., 1993; Garty and Palmer, 1997). ENac does not appear to be located in crypt cells, further confirming crypt enterocytes are most likely secretory (Greger et al., 1997). Studies have revealed that ENac also appears to be regulated by CFTR. A larger Na^+ conductance was observed in colonic mucosa from individuals with cystic fibrosis (Mall, 1999). Furthermore, $CFTR^{-/-}$ mice show an increased amiloride-sensitive short circuit current (I_{sc}) compared to WT, implicating normal CFTR function inhibits ENac absorption (Clarke and Harline, 1996). The ouabain-sensitive serosal electrogenic $3Na^+/2K^+$ ATPase removes $3Na^+$ from the cytosol and allows entry of $2K^+$. This generates an electrochemical gradient and a negative cell voltage that drives Na^+ absorption (Kunzelmann and Mall, 2002). The transport of Na^+ into the intercellular space, is followed by Cl^- and this initiates fluid movement (Greger et al., 1997). The intercellular Cl^- exits via serosal Cl^- channels or the Cl^-/HCO_3^- exchanger.

Fluid movement from the lumen into the general circulation occurs via paracellular shunting or transcellular movement via luminal and serosal aquaporins (AQP) (Wang et al., 2000). Three types of AQPs exist in the colonic mucosa, AQP3, AQP4, AQP8 (Ma and Verkman, 1999). Studies in transgenic mice have shown that loss of AQP4 results in 50 % loss of water permeability and increased stool water content (Wang et al., 2000). Conversely, loss of AQP3 (Ma et al., 2000) or AQP8 (Yang et al., 2005),

has no effect on colonic water permeability. Thus, AQP4 appears to be the primary AQP responsible for fluid absorption in the mouse.

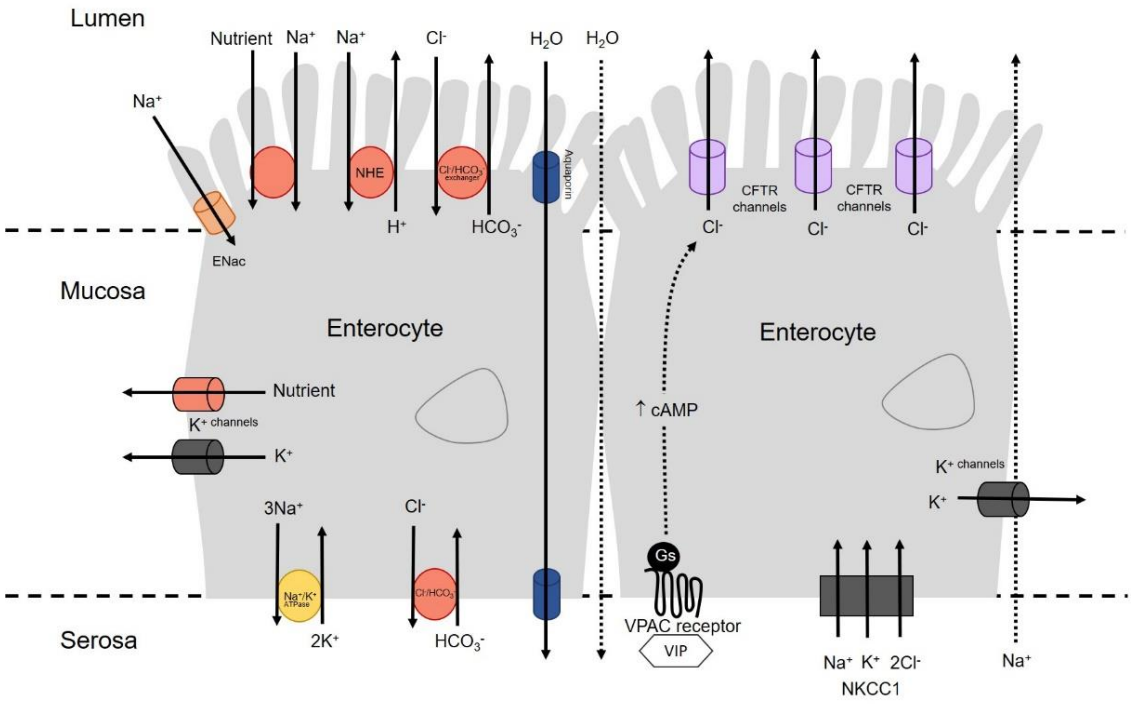


Figure 1.16: Intestinal epithelial ion transport in absorptive/secretory enterocytes. Left cell: Absorption of ingested organic solutes (glucose and amino acids) enter enterocytes via co-transporters, that couple to a sodium ion (Na^+). These nutrients exit the serosal membrane independent of ion transport. Electroneutral transport of Na^+ and chloride ions (Cl^-) occurs via Na^+/H^+ (NHE) and Cl^-/HCO_3^- exchangers. Na^+ is also absorbed via the epithelial sodium channel (ENac). In the serosal membrane, the $3Na^+/2K^+$ ATPase is electrogenic and uses ATP to drive transport of ions against their electrochemical gradient. Serosal potassium ion (K^+) channels open allowing exit of K^+ ions to prevent cell swelling. Water is absorbed via paracellular shunting or transcellular via AQPs. Right cell: The secretagogue, VIP, activates VPAC receptors that are $G_{\alpha s}$-coupled. This leads to an increase in cAMP accumulation and encourages the opening of CFTR channels, to cause Cl^- secretion. The loss of Cl^- facilitates electroneutral inward flux of Na^+, K^+ and $2Cl^-$ via the NKCC1 cotransporter. The influx of K^+ prolongs Cl^- secretion.

1.16.2 Intestinal epithelial ion secretion

Fluid secretion and absorption are balanced to prevent diarrhoea/constipation and also to maintain hydration and mucus secretion (Kunzelmann and Mall, 2002). Activation of secretory cells in the intestine with a secretagogue (e.g. VIP) increases cAMP activation and this drives the probability of CFTR opening, to cause Cl^- secretion (Figure 1.16) (Field, 2003). The CFTR channel is a member of the ATP-binding cassette protein family and dysfunction of the channel leads to cystic fibrosis (Riordan et al., 1989). Utilising *in situ* hybridisation, studies have shown CFTR mRNA expression was graded along the crypt-villus axis, with lowest expression observed at the villus tip in rat (Trezíse and Buchwald, 1991) and human (Strong et al., 1994). Thus, crypt enterocytes are predominantly secretory. CFTR is activated by $G_{\alpha s}$ or $G_{\alpha q}$ pathways to cause secretion of Cl^- (Mall et al., 1998; Barrett and Keely, 2000). In this way, CFTR has dual function in the intestine a) in the crypt enterocytes, the main function of CFTR is Cl^- secretion, and b) in the surface epithelium enterocytes, CFTR is also an important regulator of ENac and electroneutral NaCl absorption. CFTR Cl^- secretion is dependent on the serosal $Na^+K^+2Cl^-$ co-transporter (NKCC1) (Haas and Forbush III, 2000). Loss of NKCC1 in mice ($NKCC1^{-/-}$) ameliorated colonic Cl^- secretion, demonstrating NKCC1 as a key regulator of Cl^- secretion (Flagella et al., 1999) (Figure 1.16). Newly arrived intercellular K^+ from NKCC1 prevents cell shrinkage (Lang et al., 1998) and is recycled back into the circulation via serosal K^+ channels (cAMP dependent- or Ca^{2+}) or electroneutral serosal K^+/Cl^- co-transporters, to prevent cell swelling (Kunzelmann and Mall, 2002). This recycling of K^+ prolongs Cl^- secretion and NaCl absorption (Field, 2003).

1.16.3 Y receptor signalling inhibits epithelial ion transport and upper intestinal transit is increased in $PYY^{-/-}$ mice

In 1988, Cox and Cuthbert revealed PYY and NPY inhibited basal I_{sc} and VIP-induced secretory responses in rat jejunum and descending colon mucosa. These responses were absent in unstripped tissue, demonstrating the circular and longitudinal muscle provided a barrier to exogenous peptides. These anti-secretory responses were 50 % inhibited by piretanide, a NKCC1 inhibitor, suggesting the movement of Na^+, K^+ and $2Cl^-$ partially mediated the anti-secretory responses (Cox et al., 1988). Furthermore,

$^{36}Cl^-$ and $^{22}Na^+$ flux studies demonstrated the anti-secretory responses were attributed to the attenuation of Cl^- anion secretion (from serosal to mucosal side) via luminal anion channels (presumably CFTR channels) (Cox et al., 1988). Indeed, Hubel and Renquist (1986) demonstrated activation of Y receptors ($G_{\alpha i}$-coupled), reduced cAMP production and PKA activity in enterocytes. This was attributed to the attenuation of Cl^- secretion via luminal CFTR channels and the reduction of K^+ conductance, causing an anti-secretory response (Hubel and Renquist, 1986).

In early GI electrophysiology studies, the rat or guinea pig were used as the preferred species of choice. Today, the mouse is the most commonly utilised species due to the availability of transgenic and knockout mice lacking a single Y receptor, which has revealed detailed analysis of specific genes, receptors and their pharmacology and physiology (Gershon, 1999). As a result, Cox *et al.* (2001) investigated the effects of NPY, PYY and hPP in the mouse. PYY, NPY and hPP inhibited VIP-induced anion secretion in descending colon mucosa (Cox et al., 2001), as observed previously in the rat (Cox et al., 1988). The receptor selectivity of these three agonists were investigated using Y_1 (BIBO3304 or BIBP3226) an Y_2 (BIIE2046) competitive antagonists. PYY, NPY and hPP responses were Y_1-, Y_2- and Y_4-mediated, respectively (Cox et al., 2001; Tough et al., 2006). These findings were similar to those observed in descending human colon mucosa (Cox and Tough, 2002), and were confirmed by the loss of selective agonism in $Y_2^{-/-}$- (Hyland et al., 2003) and $Y_4^{-/-}$ mouse tissue (Tough et al., 2006). Studies carried out by Hyland *et al.* (2003) demonstrated Y_2 receptors were located pre-junctionally in neurons in mouse and human descending colonic mucosa, whereas Y_2 receptors were post-junctional in longitudinal smooth muscle (Hyland et al., 2003). Additionally, $PYY_{(3\text{-}36)}$ Y_2 responses were also 90 % inhibited in the presence of TTX, demonstrating a predominant neuronal component, and a relatively minor epithelial post-junctional proportion of Y_2-mediation. Importantly, Y_1, Y_2 and Y_4 signalling in the mouse and human colonic mucosa was identical, and therefore the mouse was chosen as the preferred species to use in further studies (Cox et al., 2001; Cox and Tough, 2002; Hyland et al., 2003; Tough et al., 2006). The competitive Y receptor antagonists (BIBO3304 and BIIE2046) also revealed basal Y_1 and Y_2 tone in mouse and human colonic mucosa, implicating tonic PYY-Y_1 and NPY-Y_2 activity (Hyland et al., 2003; Hyland and Cox, 2005; Tough et al., 2011). Utilising various knockout mice ($NPY^{-/-}$, $PYY^{-/-}$ and $NPY/PYY^{-/-}$) and human mucosa, Tough *et al.*

(2011) revealed Y_1 tone was predominantly TTX-insensitive mediated by Y_1 receptors (90 %) and the residual 10 % was Y_2-mediated and TTX-sensitive. Conversely, Y_2 tone was primarily Y_2-mediated and attenuated in the presence of TTX (Cox, 2008; Tough et al., 2011). Taken together, these studies showed that in the mouse, Y_1 and Y_4 responses were mainly epithelial in origin and Y_2 responses were predominantly neuronally-mediated. Thus, revealing differential expression of these three Y receptors in the intestine.

The effect of Y-receptor signalling on colonic motility *in vitro* and upper GI transit (UGIT) *in vivo* has been assessed. The Y_1 antagonist (BIBO3304) appeared to increase colonic transit, whereas the Y_2 antagonist (BIIE2046) appeared to slow colonic transit, in isolated colon *in vitro.* However, these effects were not significant. UGIT was significantly faster in $PYY^{-/-}$ mice and the transit rate in the double knockout NPY/$PYY^{-/-}$ mice was similar to WT, indicating loss of NPY negates the inhibitory effects of PYY (Tough et al., 2011). Therefore, PYY slowed colonic and upper GI transit as shown previously (Savage et al., 1987; Lin et al., 1996), presumably via the Y_1 receptor (Tough et al., 2011), whereas NPY increased colonic transit as shown previously (Chen et al., 1997; Monnikes et al., 2000). This NPY-induced increase in colonic motility was Y_2-mediated *in vivo* (Tough et al., 2011).

More recently, Cox *et al.* (2010) showed that activation of the ethanolamide receptor GPR119, with the selective GPR119 agonist, PSN632408 (Cox et al., 2010) or PSN-GPR119 (Patel et al., 2014) was PYY Y_1-mediated and glucose-sensitive in mouse and human mucosa, and improved glucose tolerance in mice. These key GPR119 studies demonstrated the benefit of targeting L cell GPR119, as a relevant therapeutic target in T2DM. This thesis further investigated GPR119 signalling and additionally interrogated novel FFA1 and FFA4 signalling in the mouse, using selective agonists and antagonists provided by AZ.

1.17 Thesis aims

At the start of this PhD, the pharmacology of the FFA1, FFA4 and GPR119 receptors in native tissue was unclear, and very few functional studies had been performed interrogating their intestine mechanisms. The first aim of this study was to determine the potencies and efficacies of novel selective agonists received from AZ (FFA1 agonists: JTT, TAK-875; FFA4 agonists: AZ423, Met-36; GPR119 agonists: Cpd.16, Cpd.42) and compare them with commercially available agonists (FFA1 agonist: TUG424; FFA4 agonist: TUG891; FFA1 and FFA4 dual agonist: GW9508; GPR119 agonist: PSN632408). Next, a tissue survey was undertaken to investigate the GI regional variations in FFA1, FFA4 and GPR119 agonism, and this allowed selection of an optimal area for future functional studies.

In descending colon mucosa, the selectivity of the FFA1-preferring antagonist, GW1100 and the FFA4 antagonist, AH-7614 was established, comparing them with the selective AZ FFA1 antagonist, ANT825. The relative abilities of each antagonist to reveal tonic FFA1 or FFA4 activity was evaluated. Additionally, each antagonist was utilised to validate the selectivity of the chosen agonists. The involvement of endogenous PYY in FFA1, FFA4 and GPR119 agonism was determined by blocking endogenous PYY activity pharmacologically, with the competitive Y_1 antagonist (BIBO3304) and Y_2 antagonist (BIIE2046). The glucose-sensitivity of these responses was evaluated as hypoglycaemia is a common adverse effect of some T2DM therapeutics, namely sulphonylureas and insulin. Moreover, the TTX-sensitivity of FFA1, FFA4 and GPR119 agonism was assessed to determine the enteric neuronal involvement in their mucosal responses.

As GLP-1 responses were not observed in FFA1, FFA4 or GPR119 agonism in the descending colon mucosal Ussing chamber studies, another tissue survey was undertaken to identify the area of colon that provided the greatest GLP-1-mediated response. In this region, GPR119 agonism was assessed in the presence of a DPPIV-inhibitor (sitagliptin), as the GPR119 receptor has a significant role in GLP-1 release in primary colonic crypt cultures and in mice *in vivo*, compared to the FFA1 and FFA4 receptors (Ekberg et al., 2016).

Physiologically, lipid metabolites from the diet concomitantly activate all three lipid-sensing receptors, FFA1, FFA4 and GPR119. Thus, potential synergistic effects of

co-agonism and triple agonism was evaluated in the descending colon. L cell triple receptor agonism was studied utilising dietary pinolenic acid, a newly characterised dual FFA1 and FFA4 agonist, and this was applied with a commercial or AZ supplied GPR119 agonist. As PYY is a known mediator of the ileal and colonic brakes, the effect of FFA1 and FFA4 agonism on upper intestinal motility and colonic motility was assessed *in vivo*.

A summary of the selective AZ compounds and commercially available agonists used in this thesis is shown in Table 1.4. Furthermore, the AZ in-house potency and selectivity data of each AZ compound is shown in Table 1.5.

	Agonist				Antagonist			
	From	Name	International Non-proprietary name	Abbreviated name used in this thesis	From	Name	International Non-proprietary name	Abbreviated name used in this thesis
FFA1	AZ	AZ13503098 AZ13667747	Fasiglifam -	TAK-875 JTT	AZ	ANT825	Compound 39	ANT825
	Commercial	Pinolenic acid GW9508 TUG424	- - -	Pinolenic acid GW9508 TUG424	Commercial	GW1100	GW1100	GW1100
FFA4	AZ	AZ13595423 AZ13552400	Compound 34 Metabolex-36	AZ423 Met-36	-	-	-	-
	Commercial	Pinolenic acid GW9508 TUG891	- - -	Pinolenic acid GW9508 TUG891	Commercial	AH-7614	-	AH-7614
GPR119	AZ	AZ13279475 AZ13457680	Compound 42 Compound 16	Cpd.42 Cpd.16	-	-	-	-
	Commercial	PSN632408	-	PSN632408	-	-	-	-

Table 1.4: Synthetic agonists and antagonists used in this thesis, which were either received from AstraZeneca or commercially bought. The table includes compound receptor target(s), other names, international non-proprietary names and the abbreviated names used in this thesis.

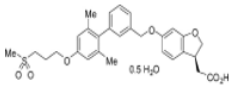
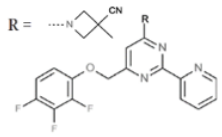
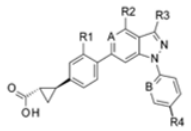
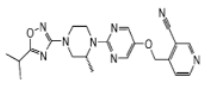
Receptor	Compound	Structure	Potency (signalling)	Selectivity (all human unless otherwise stated)
FFA1	TAK-875	 $LogD_{7.4}$: 1.44	Cell based – HEK293 or CHO cells transfected with FFA1 (measured by FLIPR). Mouse – 31 nM CHO Mouse – 182 nM HEK293 Human – 5 nM HEK293	AZ and MDS Pharma Screen (includes GPCRs, other receptors, ligand-gated ion channels, ion channels, amine transporters, enzymes) No activity at 30 µM or more against a panel of targets, including: GPR39 Cytochrome P450 FFA4 Active at 1.8 µM or more at: hPPARγ
	JTT	Not available (AZ unable to disclose)	Cell based – HEK293 cells transfected with FFA1 (measured by FLIPR). Mouse – 435 nM	AZ and MDS Pharma Screen (includes GPCRs, other receptors, ligand-gated ion channels, ion channels, amine transporters, enzymes) No activity at 30 µM or more against a panel of targets, including: Cytochrome P450 enzymes
	ANT825	 $LogD_{7.4}$: 3.3	Cell based – HEK293 cells transfected with FFA1. Stimulated with elaidic acid and measured IC_{50} in IP-one assay (time resolved fluorescence). Mouse – 81 nM Human – 165 nM Rat – 60 nM	AZ and MDS Pharma Screen (includes GPCRs, other receptors, ligand-gated ion channels, ion channels, amine transporters, enzymes) No activity at 30 µM or more against a panel of targets, including: FFA2 FFA2 (rat) Cytochrome P450 enzyme
FFA4	AZ423	 A : CH R1: H R2: H R3: Me B : CH R4: Me $LogD_{7.4}$: 3.8	Cell based – CHO cells transfected with FFA4 (FLIPR). Mouse FFA4 – 1.5 µM Human FFA4 – 2 µm Cell based – HEK293 cells transfected with FFA4 (EPIC assay – measured changes in cell shape upon ligand binding providing an EC_{50}) Mouse – 160 nM Human – 214 nM Rat – 170 nM	AZ and Cerep Pharma Screen (includes GPCRs, other receptors, ligand-gated ion channels, ion channels, amine transporters, enzymes) No activity at 10 µM or more against a panel of targets, including: NPY1 NPY2 CB1 CB2 SST1-5 FFA1 FFA1 antagonism mFFA1 FLIPR agonism – inactive. mFFA1 FLIPR antagonism – 13.6 µM Secondary pharmacology screen (EC_{50} or IC_{50}) $5HT_{2B}$ – 4.3 µM Ghrelin antagonism – 1.23 µM
GPR119	Cpd.42	 $LogD_{7.4}$: 3.3	Cell based – HEK293 transfected with GPR119 (measured cAMP time resolved fluorescence EC_{50}) Mouse – 77nM Human – 8 nM Rat – 165 nM Dog – 11 nM	AZ and MDS Pharma Screen (includes GPCRs, other receptors, ligand-gated ion channels, ion channels, amine transporters, enzymes) No activity at 10 µM or more against a panel of targets, including: NPY1 NPY2 CB1 CB2 SST1-5 FFA1 FFA1 antagonist FFA4 Active at 10 µM or more at: AChE – 73 % inhibition (IC_{50}: 3.6 µM) $5\text{-}HT_{2B}$ – 50-75 % inhibition (IC_{50}: 7.2 µM) VMAT – 66 % inhibition

Table 1.5: AstraZeneca in-house potency and signalling data of compounds used in this thesis. Abbreviations: 5-HT, serotonin; AChE, acetyl cholinesterase; cAMP, cyclic adenosine monophosphate; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; CHO, Chinese Hamster Ovary; FFA1, free-fatty acid receptor 1; FFA4, free-fatty acid receptor 4; FLIPR, fluorometric imaging plate reader; HEK, human embryonic kidney; NPY, neuropeptide Y; PPAR, peroxisome proliferator activator receptor; SST, somatostatin receptor; VMAT, vesicular monoamine transporter.

CHAPTER 2

METHODS & MATERIALS

2.1 Mucosal preparation and measurement of vectorial ion transport (short-circuit current (I_{sc}))

Mice (C57BL/6J, 12-20 weeks old, 20-30 g, female or male) were procured from Charles River Laboratories (Margate, UK) had free access to standard chow (Rat and Mouse No 3 breeding diet, Special Diets Service, Braintree, UK) and water *ad libitum*. This species and strain was used because the Y_1, Y_2 and Y_4 expression patterns in colonic mucosa is the same as that observed in human (Cox and Tough, 2002; Hyland et al., 2003; Tough et al., 2006; Tough et al., 2011). Mice were housed in a convention unit, in open top conventional cages with Lignocel poplar bedding material along with the appropriate environment enrichment. A maximum of five mice were housed in a single cage. Housing rooms were maintained at 20-24 ˚C, humidity 55 % ± 10 % and 12/12 h light and dark cycles, with lights on from 07.00 am. All animal care and experimental procedures complied with the Animals (Scientific procedures) Act 1986 and were approved by the local ethics committee. Mice were killed by cervical dislocation and the whole colon or specific area of GI tract was dissected (noting the proximal and distal regions) and placed in fresh Krebs-Henseleit (KH), with the following composition (in mM); NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, and D-glucose 11.1.

First, the mucosal sheets were prepared by cutting along the mesenteric line and carefully removing luminal contents. The tissue was carefully pinned (mucosal side-down) and the overlying smooth muscle layers (both circular and longitudinal muscle with the associated myenteric plexus) were removed by micro-dissection. The mucosal preparation was cut into adjacent pieces (~0.5 cm); the descending colon (i.e. transverse-distal colon to rectum) routinely provided 6 adjacent pieces and the ascending colon (i.e. cecal junction to transverse colonic regions) providing 4 preparations. Each mucosal preparation was mounted between two halves of an Perpex Ussing chamber (exposed area, 0.14 cm^2) within 30 mins of excision from the mouse, bathed in 5 ml of oxygenated KH (95 % O_2/ 5 % CO_2) on each side of the reservoir at 37 ˚C, voltage clamped at 0 mV and the resultant basal I_{sc} was measured (in $\mu A.cm^{-2}$) continuously on a chart recorder (Kipp and Zonen, Lincoln, UK), as described previously (Cox et al., 2010) (Figure 2.1). The Ussing chambers, DVC1000 and the amplifiers were purchased from WPI UK, Hitchin, Herts, UK. The mounted mucosal preparations, consisting of mucosa, submucosa and submucosal innervation,

created a barrier between the ap and bl sides of the reservoir. This orientation of the mucosal preparation allowed measurement of net electrogenic anion secretion, as an increase in I_{sc}.

Once the I_{sc} had stabilised (15 min), a 1 mV pulse was applied and the I_{sc} deflection was evaluated to measure the transepithelial resistance ($\Omega.cm^2$), calculated using Ohms law (transepithelial resistance = voltage/current). Once the mucosal preparations had equilibrated and the basal I_{sc} had stabilised, agonist or antagonist additions were made to either the ap or bl reservoirs.

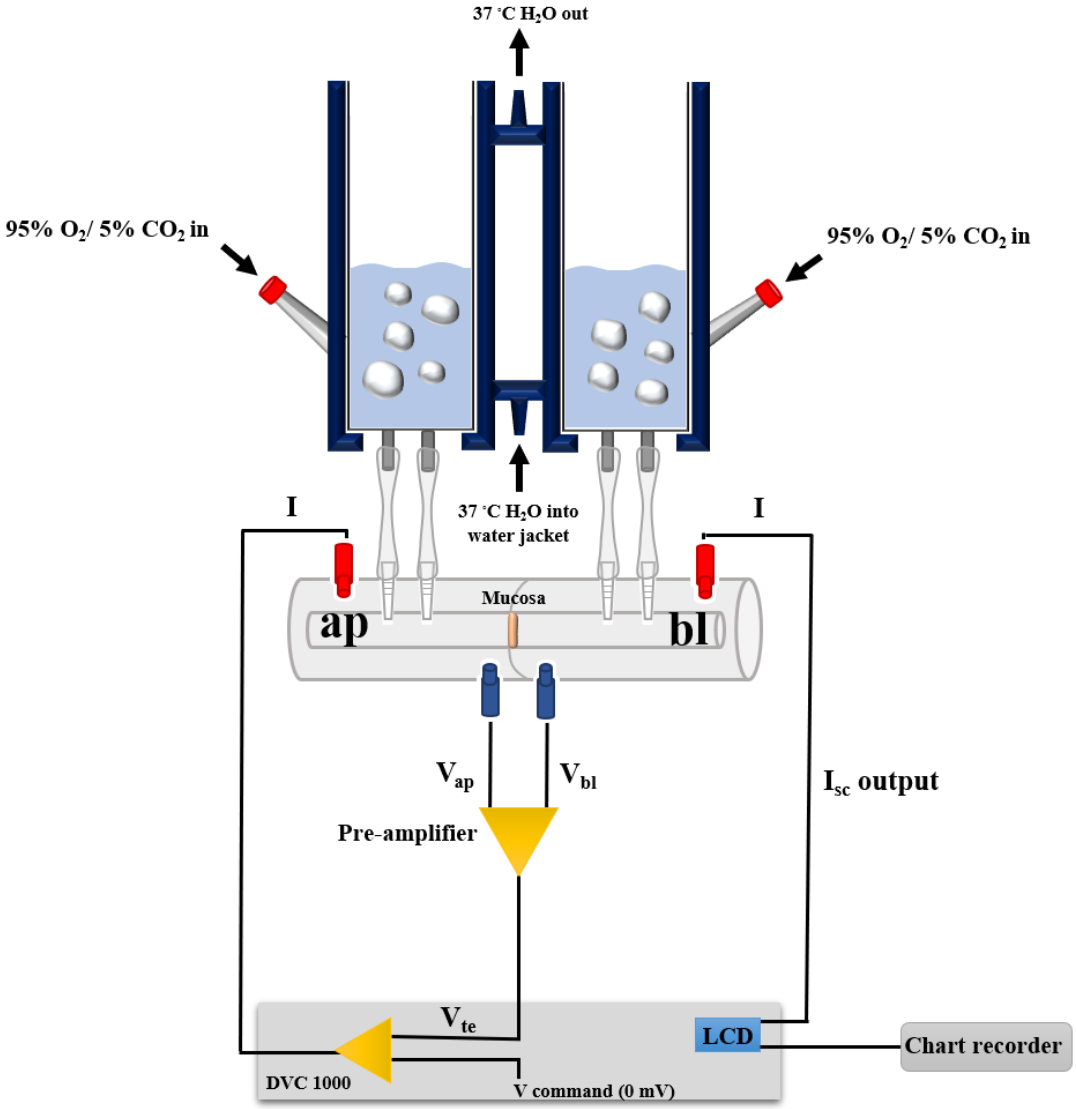


Figure 2.1: The Ussing chamber set-up. A mucosal sheet is mounted in the middle of the Ussing chamber, voltage-clamped at 0 mV and bathed in 5 ml of oxygenated KH, on each side of the reservoir. This set-up allows interrogation of receptors on the apical (ap) and basolateral (bl) sides of the mucosa. The resultant change in I_{sc} from net movement of electrogenic ion transport is measured and recorded on the chart recorder.

2.2 Sidedness, potency and efficacy of the FFA1, FFA4 and GPR119 agonists

Descending colon mucosal preparations were pretreated with VIP (30 nM unless otherwise stated, bl) to provide a degree of vectorial epithelial ion secretion upon which subsequent anti-secretory signalling was more readily observed (as optimised previously; Tough et al. 2006; Cox et al. 2010). Once VIP responses reached a maximum, the commercial available FFA1 agonist (TUG424), FFA4 agonist (TUG891) or the dual agonist (GW9508) was added to either reservoir, to determine the sidedness of these responses.

Single apical additions of the commercially available FFA1, FFA4 and GPR119 agonists (namely TUG424, TUG891, GW9508, PSN632408) and the selective agonists received from AZ (JTT, TAK-875, Met-36, AZ423, Cpd.16 and Cpd.42) were used to construct single addition concentration-response curves, comparing the commercial and selective agonists. The single EC_{50} values for each agonist were calculated. The non-linear regression formula used to fit each curve was as follows; y = min response + (max response – min response/ 1 + 10^(LogEC_{50} – x) * Hill slope), where y = response and x = Log_{10}[agonist](M). For each curve the minimum response was set to 0µA and the Hill slope was allowed to vary. Overall range of Hill slopes were -1.6 to -0.4; the commercial GPR119 agonist, PSN632408 possessed the most negative cooperativity (Hill slope of -1.6). PYY (10 nM unless otherwise stated, bl) was added finally as an internal control for 10 min. Changes in I_{sc} to each agonist were pooled and converted to $\mu A.cm^{-2}$.

Utilising an EC_{80} concentration of the selected FFA1 agonist, TAK-875 (200 nM); the FFA4 agonist, Met-36 (100 nM) and the GPR119 agonist, Cpd.42 (100 nM), the sidedness of their responses was assessed in descending colon mucosa. Agonists were applied to the ap or bl sides of the reservoir after VIP, and these responses were recorded for 20 min, followed by the addition of PYY.

2.3 Tetrodotoxin-sensitivity of FFA1, FFA4 and GPR119 responses and their regional variation in the GI tract, on basal I_{sc} and in the presence of VIP

Once the EC_{50} values were established, the FFA1 (TAK-875), FFA4 (Met-36) and GPR119 (Cpd.42) agonists were selected to use in further studies. To determine

whether their responses were neuronally-mediated or epithelial in origin, EC_{80} concentrations of each agonist were selected. The descending colon mucosal preparations were pretreated with the voltage-gated sodium channel neurotoxin, TTX (100 nM, bl) or vehicle (distal water, dH_2O), 15 min prior to VIP. Subsequently, the agonists were added, and their responses were monitored for 20 min, followed by PYY.

In the next experiment, the region of the GI tract that provided the greatest FFA1 (TAK-875), FFA4 (Met-36) or GPR119 (Cpd.42) response on basal I_{sc} and after VIP was identified. Responses were evaluated in the mid-ileum, terminal-ileum, ascending colon, and descending colon. Finally, exogenous PYY was used as an internal control.

2.4 Regional variation of Ex4-mediated GLP-1 responses in the colon

The colon was cut into eight equal sized pieces from the caecal junction, providing ascending colon (AC1-AC3), transverse colon (designated as ascending-transverse colon, ATC and descending transverse colon, DTC) and descending colon (DC3-DC1). DC1 was closest to the rectum. The mucosae were pretreated with the GLP-1 antagonist, Ex(9-39) (1 µM, bl) or vehicle (dH_2O) for 20 mins, prior to a single concentration of the GLP-1 agonist, Ex4 (100 nM, bl). The internal controls, VIP and PYY were added at the end of the experiment to increase and lower the I_{sc}, respectively.

2.5 Evaluating GPR119 agonism in the presence of the DPPIV inhibitor, sitagliptin in the ascending and descending colon

The ascending colon (AC1 - AC2) and the descending colon (DC2 – DC1) were pretreated with sitagliptin (1 µM, bl) or vehicle control (dH_2O) for 15 mins. After VIP, the PSN632408 (10 µM, ap) response was assessed in the presence and absence of sitagliptin. These latter responses were recorded for 20 mins, followed by the application of Ex4 and exogenous PYY.

2.6 Establishing the selectivity of FFA1, FFA4 and GPR119 agonism using FFA1 and FFA4 antagonists

Once the basal I_{sc} levels had stabilised, varying concentrations (1 nM–10 µM) of the FFA1 antagonist, ANT825 or FFA4 antagonist, AH-7614 (1 nM–10 µM) were added to each descending colon mucosal preparation for 10-15 min. To establish IC_{50} values of ANT825 and AH-7614, VIP was applied followed by the FFA1 agonist, JTT (300 nM, ap) or FFA4 agonist, Met-36 (100 nM), respectively. The consequent reductions in I_{sc} were recorded and converted to $\mu A.cm^{-2}$. PYY was added as an internal control.

To show FFA1 selectivity, the mucosae were pretreated with the commercially available FFA1 antagonist (GW1100), or the AZ FFA1 antagonist (ANT825) or vehicle (0.1 % dimethyl sulfoxide (DMSO)) for a 10 min period, prior to VIP application. The commercially available agonists, TUG424 (100 nM), TUG891 (100 nM) and GW9508 (1 µM) were tested initially in the presence or absence of each FFA1 antagonist. Later, the selectivity of the AZ agonists, TAK-875 (200 nM), Met-36 (100 nM) and Cpd.42 (100 nM) were examined.

To investigate FFA4 selectivity, the descending colon mucosa was pretreated with a combination of the FFA1 (ANT825, 10 µM, ap) and FFA4 antagonists (AH-7614, 10 µM, ap) or the respective vehicle control for 10-15 min, followed by VIP. The selectivity the dual agonist, GW9508 (1 µM, ap) was assessed and its response was recorded for 20 min. Furthermore, the TAK-875 response (FFA1, 200 nM, ap) was evaluated in the presence of AH-7614 alone. The ability of AH-7614 (10 µM) and ANT825 (10 µM) to reveal tonic FFA1 and FFA4 activity was assessed on basal I_{sc} (over a 20 min period) in the presence of the combined competitive Y_1 antagonist (BIBO3304 (300 nM)) and Y_2 antagonist (BIIE2046 (1 µM)), or vehicle (0.013 % DMSO) in descending colon mucosal preparations.

2.7 Y_1 and Y_2 receptor antagonist (BIBO3304 and BIIE0246) studies

Descending colon mucosal preparations were pretreated with previously optimised concentrations of the Y_1 antagonist, BIBO3304 (BIBO, 300 nM, bl) or the Y_2 antagonist, BIIE0246 (BIIE, 1 µM, bl) or both, and their respective DMSO (0.003-0.1 %) controls. After 10-15 min, VIP was applied and once this response had stabilised,

a single apical concentration of the FFA1, FFA4 or GPR119 agonist was added, and the consequent reductions in I_{sc} were converted to $\mu A.cm^{-2}$. PYY and the α_2-adrenoceptor agonist, UK14,304 (1 µM) were used as internal controls.

2.8 Mucosal glucose-sensitivity of FFA1, FFA4 and GPR119 agonism

Excised whole colon was placed in fresh glucose-free KH with the following composition (in mM); NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, and D-mannitol 11.1. Descending colon mucosal preparations were micro-dissected as described above (section 2.1), placed in Ussing chambers and were bathed in KH containing glucose on both sides (control) or in KH containing the glucose replacement, D-mannitol (at 11.1 mM) on the ap side only. Mucosal basal I_{sc} levels were allowed to stabilise before the addition of VIP. Subsequently, a single apical addition of the FFA1, FFA4 or GPR119 agonist was added and consequent reductions in I_{sc} were recorded and converted to $\mu A.cm^{-2}$. Phloridzin (50 µM, ap) was used as an internal control to block apically located SGLT1, and this electrogenic activity should be impaired when mannitol replaces glucose apically. When investigating FFA1 and FFA4 agonism in the presence of low or high glucose; the glucose concentration was altered (5 mM or 25 mM glucose in the KH) on both the ap and bl sides simultaneously.

2.9 Inhibition of mucosal glucose transport via blockade of SGLT1 and GLUT2

Descending colon mucosal preparations were pretreated with a combination of the SGLT1 inhibitor, phloridzin (50 µM, ap) and the GLUT2 inhibitor, phloretin (0.1 mM, ap + bl) or vehicle (dH_2O) for 15 mins. Phloretin blocks apical and basolateral expressed GLUT2 (Gorboulev et al., 2012; Mace et al., 2012). After VIP, the FFA1 agonist (TAK-875, 200 nM, ap), the FFA4 agonist (Met-36, 100 nM, ap) or the GPR119 agonist (Cpd.42, 100 nM, ap) was added and their responses were recorded for 20 min prior to PYY addition.

2.10 Co-agonism of L cell FFA1, FFA4 or GPR119 receptors using the selective AZ agonists

First, the I_{sc} was raised with VIP in the descending colon mucosal preparations. Next, agonism of FFA1, FFA4 or GPR119 with a selective agonist was evaluated alone or in combination with one another (over a 20 min period). Initially, agonism of FFA1 (TAK-875, 1 µM, ap) and FFA4 (Met-36, 100 nM, ap) was examined. Subsequently, FFA1 and GPR119 agonism (Cpd.16, 1 µM, ap) was assessed followed by agonism of FFA4 and GPR119 in a separate mucosal preparation. Finally, PYY was added at the end of the experiment.

2.11 L cell GPR119 cross-desensitisation studies

Descending colon mucosal preparations were pretreated with a high concentration of the GPR119 agonist, PSN632408 (30 µM, ap) or vehicle (95 % ethanol, ap) for 2 min, prior to raising the I_{sc} with VIP. Following the pretreatment, the preparations were apically treated with the GPR119 agonist, PSN632408 (30 µM) or Cpd.42 (100 nM), or the FFA1 agonist, TAK-875 (200 nM) or the FFA4 agonist, Met-36 (100 nM). FFA1, FFA4 and GPR119 agonism was assessed for desensitisation, in the presence of 30 µM PSN632408. Responses to each selective agonist were monitored for 20 mins prior to the application of PYY.

2.12 Establishing the potency and pharmacology of pinolenic acid

First, to investigate the pharmacology of the commercially available dual agonist, pinolenic acid, a concentration-response curve was constructed to establish its EC_{50} value. Descending colon mucosal preparations were pretreated with VIP, followed by varying concentrations of pinolenic acid (100 nM – 10 µM, ap). These responses were monitored for 20 min. Finally, exogenous PYY was added as the internal control.

Next, having established the EC_{50}, the pharmacology of the pinolenic acid response was investigated. Descending colon mucosal preparations were pretreated with TTX (100 nM, bl), or ANT825 (10 µM, ap), or a combination of both FFA1 and FFA4 antagonists (ANT825 (10 µM, ap)+AH-7614 (10 µM, ap)), or the combination of the

competitive Y_1 and Y_2 antagonists (BIBO3304 (300 nM, bl) + BIIE2046 (1 µM, bl)) or their respective vehicle controls (dH_2O, or 0.1 % DMSO, or 0.013 % DMSO) for 15 mins, prior to raising the I_{sc} with VIP. The pinolenic acid (1 µM, ap) response was monitored in the presence and absence of each pretreatment for 20 min, followed by the addition of PYY.

2.13 Triple agonism of L cell FFA1, FFA4 and GPR119 receptors in the descending colon

L cell triple receptor agonism was investigated utilising the dual FFA1 and FFA4 agonist, pinolenic acid (1 µM, ap) and a commercially available GPR119 agonist (PSN632408, 10 µM, ap) or the selective AZ GPR119 agonist (Cpd.16, 1 µM, ap), in descending colon mucosal preparations. Initially, the I_{sc} was raised with VIP and the absolute I_{sc} level was recorded at the point of agonist addition. Triple agonism was monitored for 20 mins prior to the addition of exogenous PYY.

2.14 Measurement of faecal pellet propulsion in vitro

The colon (from the caeco-colonic junction to the rectum) was excised, photographed (t = 0 min) and bathed in KH at 37˚C with either vehicle (0.1% DMSO) or agonist (300 nM TUG424, TUG891, TAK-875 or Met-36). After 20 min (t = 20 min) each colon length was re-photographed, and the distance travelled by the remaining pellets was measured from the rectum, as described previously (Tough et al., 2011). The effects of chosen agonists on colonic transit were compared in the presence and absence of the FFA1 antagonist, GW1100 (10 µM). In these experiments, the colon was bathed in vehicle (0.1 % DMSO) or antagonist, photographed at 20 min, and then bathed in KH containing the agonist of choice for another 20 min. The colon was re-photographed at t = 40 min. The pellet movement was measured as the mean distance travelled relative to the total colonic length and was used to calculate the % colonic transit.

2.15 Measurement of colonic bead excretion in vivo

Mice were acclimatised to handling 3 days prior to experimentation and were fasted 16 h prior to the procedure (at ~ 5.30 pm), although water was provided *ad libitum*. Plasma glucose was tested before and after fasting by tail prick, utilising an AVIVA glucose monitoring kit. Distal colonic propulsion was measured according to the methods described by Forbes *et al.* (2012). One hour after administration of vehicle or drug (FFA1 agonist, TAK-875 (27 mg/kg); FFA4 agonist, Met-36 (6 mg/kg, 50 mg/kg), FFA1 antagonist, ANT825 (29 mg/kg)) or the positive control, loperamide hydrochloride (HCl, 10 mg/kg (Myagmarjalbuu et al., 2013)) by oral gavage (*po*) or i.p., mice were placed under 4-5 % isoflurane anaesthesia (Isoflurane-VET, Merial Animal Health Ltd, Harlow, UK) and a 2 mm bead was inserted 2 cm intrarectally into the distal colon using blunt tubing (Portex, 1.7 × 0.4 mm). The mice were subsequently placed into a grid bottom cage, monitored and the time to bead expulsion was measured. Once the bead was excreted, the mouse was killed by cervical dislocation. All drugs were suspended in their respective vehicles and sonicated for 30 min.

To assess the total plasma concentration of TAK-875 (27 mg/kg, *po*) and Met-36 (50 mg/kg, *po*) was consistent with AZ in-house data, a single blood sample was taken immediately after cervical dislocation via cardiac puncture, which was approximately 70 min after each agonist was administered. Blood samples were stored in Li-heparin-coated collection tubes provided by AZ (M. Persson). These blood samples were centrifuged (1200, rpm for 20 min, personal communication, M. Persson) to obtain 50 µl of plasma and thereafter stored at -20˚ C, until they were shipped to AZ (Mölndal) for further analysis.

2.16 Measurement of upper GI transit in vivo

Mice were acclimatised to handling 3 days prior to experimentation and were fasted for 16 h prior to testing (at ~ 5.30 pm). TAK-875 (27 mg/kg), Met-36 (50 mg/kg), ANT825 (29 mg/kg), their respective vehicles or the positive control, loperamide HCl (10 mg/kg) were administrated via i.p. 60 min prior to the procedure. A charcoal meal (10 % plant charcoal in 5 % gum acacia (Tough et al. 2011)) was given by intragastric gavage. 30 min later the animal was killed by cervical dislocation and the

small intestine was isolated from the pyloric to ileocecal junctions. Upper GI transit (UGIT), encompassing gastric emptying and small intestinal motility, was measured as a percentage of the distance travelled by the charcoal relative to the total length of the small intestine, as optimised previously (Forbes et al. 2012).

2.17 Statistical analysis

Pooled responses (as $\mu A.cm^{-2}$) are expressed as mean ± 1SEM from the number of observations described, using GraphPad Prism version 7.0 (GraphPad Prism Inc., La Jolla, CA, USA). An initial priori power analysis calculation was carried out (unpaired two-tailed *t*-test, $\alpha = 0.05$ and Power = 0.80 (80%)) using G*Power version 3.0.10. The power analysis indicated a power of *n*=5-6 was adequate for *in vitro* mucosal studies. Single comparisons between groups of data were analysed by unpaired, two-tailed Student's *t*-tests. Multiple comparisons were analysed by one-way ANOVA with *post hoc* Dunnett's test (in the presence of vehicle control) or Bonferroni's *post hoc* test. $P \leq 0.05$ was considered statistically significant. A mixture of male and female mice was used in this study, as no differences in FFA1, FFA4 or GPR119 responses were observed between sexes. The application of agonist/antagonist treatments to each mucosal preparation was randomised using the Latin square design technique. This guaranteed the same GI mucosal preparation did not receive the same treatment in each experimental repeat, over the duration of 4-5 experiments. In the *in vivo* studies, the vehicle or drug treatments were alternated in each cohort of mice to ensure each consecutive mouse received a different agonist/antagonist or vehicle. Any discrepancies between n number cohorts were due to the loss of a single mucosal preparation from a single animal *in vitro* or removed when the result was two standard deviations from the mean *in vitro* and *in vivo*.

2.18 Materials

FFA1 agonists (JTT and TAK-875), FFA4 agonists (AZ423 and Met-36), GPR119 agonists (Cpd.42 and Cpd.16) and the FFA1 antagonist (ANT825) were obtained from AstraZeneca, Gothenburg, Sweden. Stock solutions of drugs were dissolved in neat DMSO (at 10^{-2} M or 10^{-3} M). Initial 1:10 dilutions were in neat DMSO and subsequent

serial dilutions were in dH_2O (excluding the FFA1 agonist, TAK-875 and the FFA4 agonist, AZ423, where all serial dilutions were performed in distilled water). The commercially available full GPR119 agonist, PSN632408 (tert-butyl 4-((3-(pyridin-4-yl)-1,2,4-oxadiazol-5-yl)methoxy)piperidine-1-carboxylate) was purchased from Cayman Chemicals (Ann Arbor, USA) and diluted in 95 % ethanol. BIBO3304, (*N*-[(1*R*)-1-[[[[4[[(Aminocarbonyl)amino]methyl]phenyl]methyl]amino]carbonyl]-4-[(aminoiminomethyl)amino]butyl]-α-phenyl-benzeneacetamide ditrifluoroacetate), BIIE0246, (*N*-[(1*S*)-4-[(Aminoiminomethyl)amino]-1-[[[2-(3,5-dioxo-1,2-diphenyl-1,2,4-triazolidin-4-yl)ethyl]amino]carbonyl]butyl]-1-[2-[4-(6,11-dihydro-6-oxo-5*H*-dibenz[*b*,*e*]azepin-11-yl)-1-piperazinyl]-2-oxoethyl]-cyclopentaneacetamide), GW9508 (4-[[(3-Phenoxyphenyl)methyl]amino]benzenepropanoic acid), TUG424, (3-(4-(o-Tolylethynyl)phenyl)propanoic acid), TUG891 **(**4-[(4-Fluoro-4'-methyl[1,1'-biphenyl]-2-yl)methoxy]-benzenepropanoic acid), the FFA4 antagonist, AH-7614 (4-Methyl-*N*-9*H*-xanthen-9-yl-benzenesulfonamide) were purchased from Tocris Bioscience (Bristol, UK). GW1100 (4-[5-[(2-ethoxy-5-pyrimidinyl)methyl]-2-[[(4-fluorophenyl)methyl]thio]-4-oxo-1(4H)-pyrimidinyl]-benzoic acid,ethyl ester) was bought from Cambridge Bioscience (Cambridge, UK). VIP and PYY were purchased from Cambridge Bioscience (Cambridge, UK) and TTX from Abcam (Cambridge, UK). VIP, PYY and TTX were dissolved in distilled water. Ex4 and Ex(9-39) were bought from Generon (Slough, UK) and were also diluted in distilled water. All peptide stocks were stored at -20˚ C until required and underwent a single freeze-thaw cycle. Phloridzin (1-[2-(β-D-glucopyranosyloxy)-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl]-1-propanone), phloretin ((E)-1-[2,4-dihydroxy-6-[[(2R,3S,4R,6R)-3,4,6-trihydroxyoxan-2-yl]methoxy]phenyl]-3-(4-hydroxyphenyl)prop-2-en-1-one) and UK 14,304 were purchased from Sigma-Aldrich (Dorset, UK) and it too was dissolved in distilled water. Sitagliptin was bought from LKT Laboratories (Minnesota, USA) and was diluted in distilled water. Pinolenic acid was purchased from Cayman Chemicals (Ann Arbor, USA) and was diluted in 95 % ethanol. The following *in vivo* vehicles were all suspended in warm saline, namely methylcellulose and polyvinylpyrrolidinone (PVP) from Sigma-Aldrich (Dorset, UK), sodium dodecyl sulphate (SDS) from VWR International (Leicestershire, UK), hydroxylpropyl methylcellulose (HPMC) from Alfa Aesar (Lancashire, UK), Tween-80 from Boston BioProducts (Ashland, USA). Loperamide HCl was purchased from LKT Laboratories (Minnesota, USA) and was suspended in 0.5 % methylcellulose.

CHAPTER 3

RESULTS

3.1 The sidedness and efficacy of commercially available FFA1 and FFA4 agonists in descending colon mucosa

First, basal resistance and I_{sc} levels after stabilisation of the descending colon mucosal preparations were analysed and were within the ranges published previously (Figure 3.1A) (Tough et al. 2011). The activity of the non-selective FFA1 and FFA4 agonist, GW9508, as well as the TUG compounds (TUG424 and TUG891) added apically or basolaterally, induced a biphasic change in I_{sc}. The 1˚ responses were recorded within 0-5 min of agonist addition and were due to vehicle, DMSO (Figure 3.1B). Extrapolation of the waning VIP secretory response revealed the 1˚ monophasic response observed in the presence of vehicle (Figure 3.1B) was similarly observed in the presence of agonist (Figure 3.1C). The 2˚ reductions in I_{sc} after each agonist addition, occurred within 10-15 min (Figure 3.1C). When these reductions in I_{sc} were compared, there were no significant differences between the response sizes and the kinetics between the apical and basolateral responses to each agonist (Figure 3.1D).

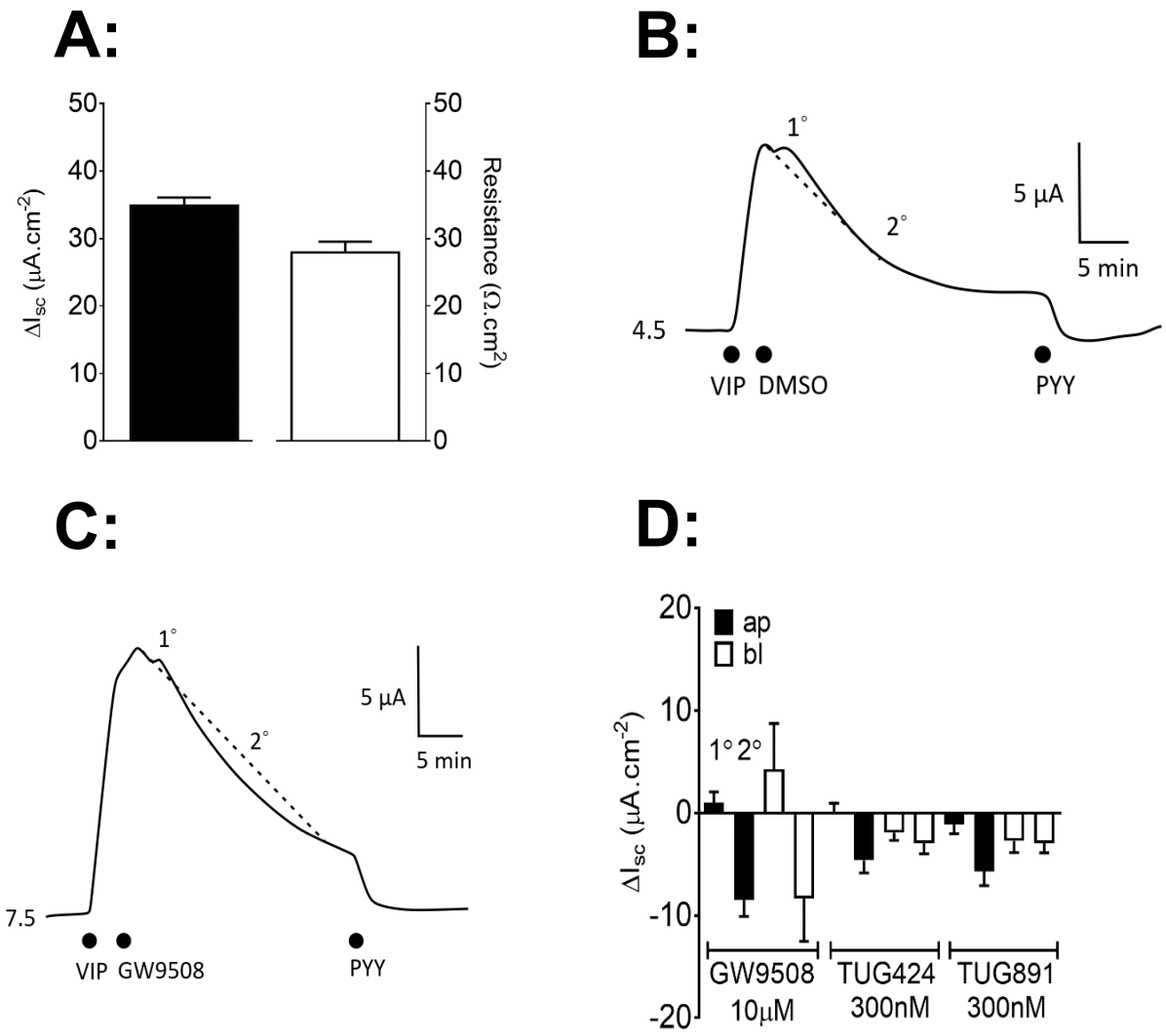


Figure 3.1: Sidedness of FFA1 and FFA4 responses in descending colon mucosa. In A: basal I_{sc} and resistance values after stabilisation of mucosal preparations (*n*=20). In B: representative trace showing monophasic nature of vehicle control, DMSO (0.1 %) in descending colon mucosa, after VIP (30 nM) and followed by PYY (10 nM). In C: representative trace showing the biphasic nature of the apical GW9508 (10 μM) response in descending colon mucosa, after VIP (30 nM) and followed by PYY (10 nM). In D: pooled data showing the primary (1˚) and secondary (2˚) responses to apical (ap) or basolateral (bl) additions of GW9508 (*n*=5), TUG424 (*n*=5) or TUG891 (*n*=5) after VIP pretreatment. Bars represent the mean ± 1SEM.

3.2 *The potency and efficacy of AZ FFA1, FFA4 and GPR119 agonists in descending colon mucosa*

The AZ selective FFA1 agonists, TAK-875 and JTT; FFA4 agonists, Met-36 and AZ423 or GPR119 agonists, Cpd.16 and Cpd.42 were added apically and induced a biphasic I_{sc} response, in descending colon mucosal preparations. The rapid first I_{sc} component witnessed within the first 5 min was attributed to the vehicle, 0.1 % DMSO (2.7 ± 0.5 µA.cm^{-2}, *n*=12) when compared with the same component for JTT (1.7 ± 0.9 µA.cm^{-2}, *n*=5), Met-36 (3.2 ± 1.7 µA.cm^{-2}, *n*=4), Cpd.16 (1.5 ± 0.7 µA.cm^{-2}, *n*=7) and Cpd.42 (3.7 ± 1.4 µA.cm^{-2}, *n*=5) or the vehicle 0.01 % DMSO (3.0 ± 1.1 µA.cm^{-2}, *n*=6) when compared with TAK-875 (2.5 ± 1.3 µA.cm^{-2}, *n*=6) and AZ423 (4.6 ± 1.4 µA.cm^{-2}, *n*=6).

Only the later reductions in I_{sc} (2° component) to varying concentrations of the TUG compounds, GW9508 and the AZ selective FFA1, FFA4 and GPR119 agonists are shown in Figure 3.2A-C. The reductions in I_{sc} to each FFA1, FFA4 or GPR119 agonist were concentration-dependent. When comparing the FFA1 agonists, TUG424 and TAK-875 were similarly potent and efficacious, and their EC_{50} values were 57.1 nM (24.7 – 131.8 nM) and 67.6 nM (30.6 – 149.4 nM), respectively. JTT was more potent (EC_{50} of 20.7 nM (12.7 – 34.0 nM)) than TAK-875 and TUG424, however they all exhibited similar efficacy (Figure 3.2A). GW9508 was less potent with an EC_{50} value of 354.8 nM (191.6 – 656.8 nM) and its maximal response appeared to be slightly larger than the maximal responses of the TUG agonists, the selective FFA1 agonists (Figure 3.2A) and the FFA4 agonists (Figure 3.2B). In this way, GW9508 appeared to be a dual agonist in comparison to the selective FFA1 and FFA4 agonists.

The FFA4 selective agonists, Met-36 (EC_{50} of 15.4 nM (7.9 – 30.4 nM)) and AZ423 (EC_{50} of 17.3 nM (3.6 – 83.8 nM)) were similarly potent and efficacious (Figure 3.2B). The commercially available FFA4 agonist, TUG891 (EC_{50} of 62.5 nM (24.1 – 162.3 nM)) was similarly potent to the FFA1 agonist, TUG424 and exhibited similar efficacy to Met-36 and AZ423 (Figure 3.2B).

The GPR119 agonists, Cpd.16 and Cpd.42 were similarly potent and efficacious and their EC_{50} values were 23.4 nM (10.2 – 53.4 nM) and 49.3 nM (6.4 – 382.6 nM) (Figure 3.2C). The anti-secretory responses of Cpd.16 and Cpd.42 were compared with the commercially available and proven full GPR119 agonist, PSN632408. In this

study, PSN632408 decreased the I_{sc} and provided an EC_{50} value of 4.8 µM (2.2 – 10.6 µM). The PSN632408 maximal response was more efficacious than the maximal response to Cpd.16 ($P \leq 0.003$) and Cpd.42 ($P = 0.093$), but displayed a lower potency compared to both AZ GPR119 agonists. The Cpd.16 and Cpd.42 responses appeared partial when compared with the PSN632408 response (Figure 3.2C).

The sidedness of FFA1, FFA4 and GPR119 agonism was assessed utilising EC_{80} concentrations of the selected AZ FFA1 agonist, TAK-875; the FFA4 agonist, Met-36 and the GPR119 agonist, Cpd.42 (Figure 3.2 continued). There were no significant differences between ap and bl FFA1 (D), FFA4 (E) or GPR119 (F) response sizes or their kinetics (Figure 3.2 continued, D-F). Furthermore, the absolute I_{sc} levels before each agonist was applied were no different between mucosal preparations receiving ap or bl treatment (Figure 3.2 continued, D-F insets).

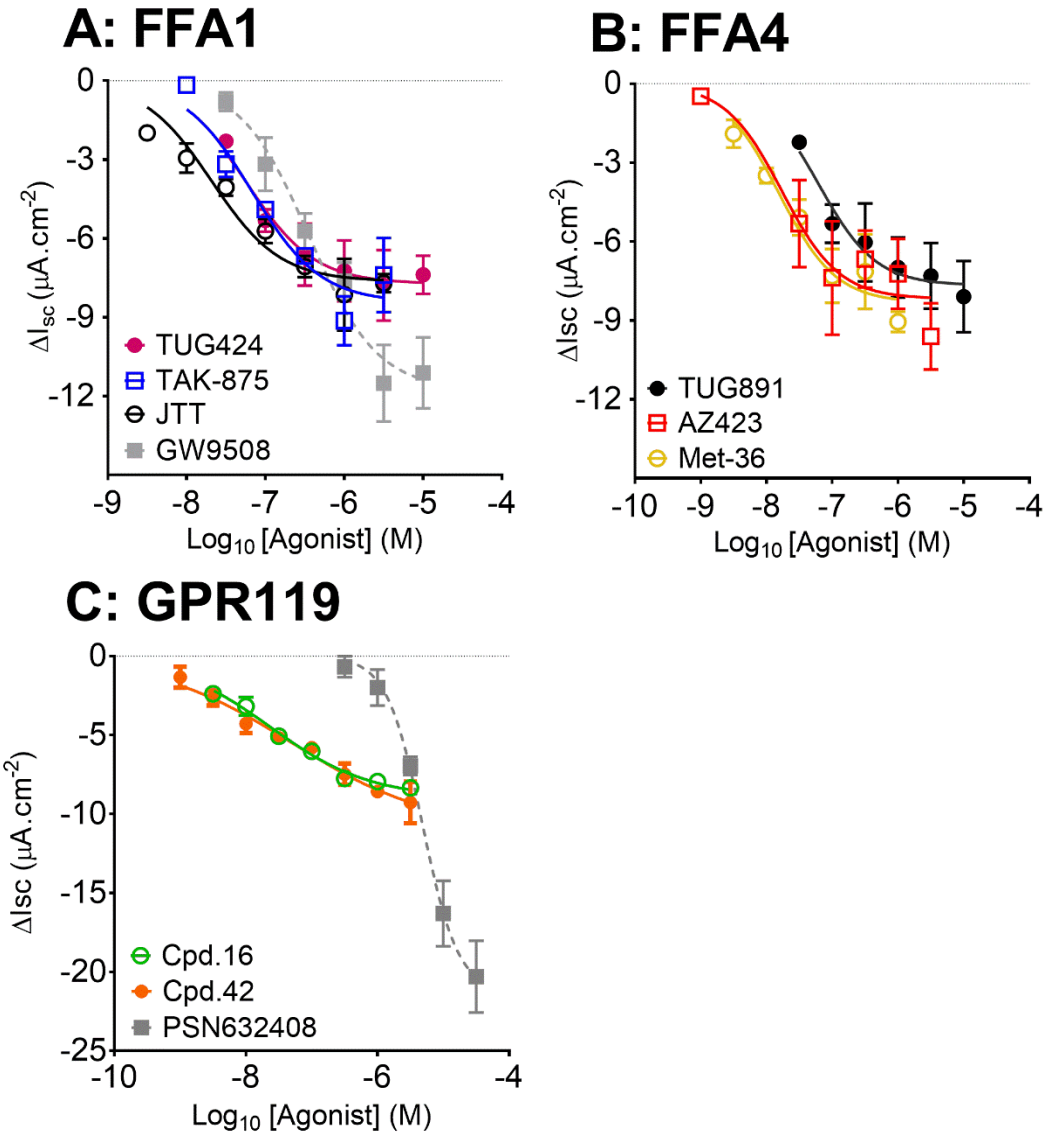


Figure 3.2: FFA1, FFA4 and GPR119 responses in descending colon mucosa. Concentration-response curves for the 2˚ apical effects of FFA1 agonists: TUG424 (*n*=5-6), TAK-875 (*n*=5-8) and JTT (*n*=5), compared with GW9508 (*n*=5) in A; for FFA4 agonists: TUG891 (*n*=5), AZ423 (*n*=5) and Met-36 (*n*=5) in B, and for GPR119 agonists, Cpd.16 (*n*=5-8), Cpd.42 (*n*=5-7) and PSN632408 (*n*=6-7, (Cox et al., 2010)) in C. Points represent the mean ± 1SEM.

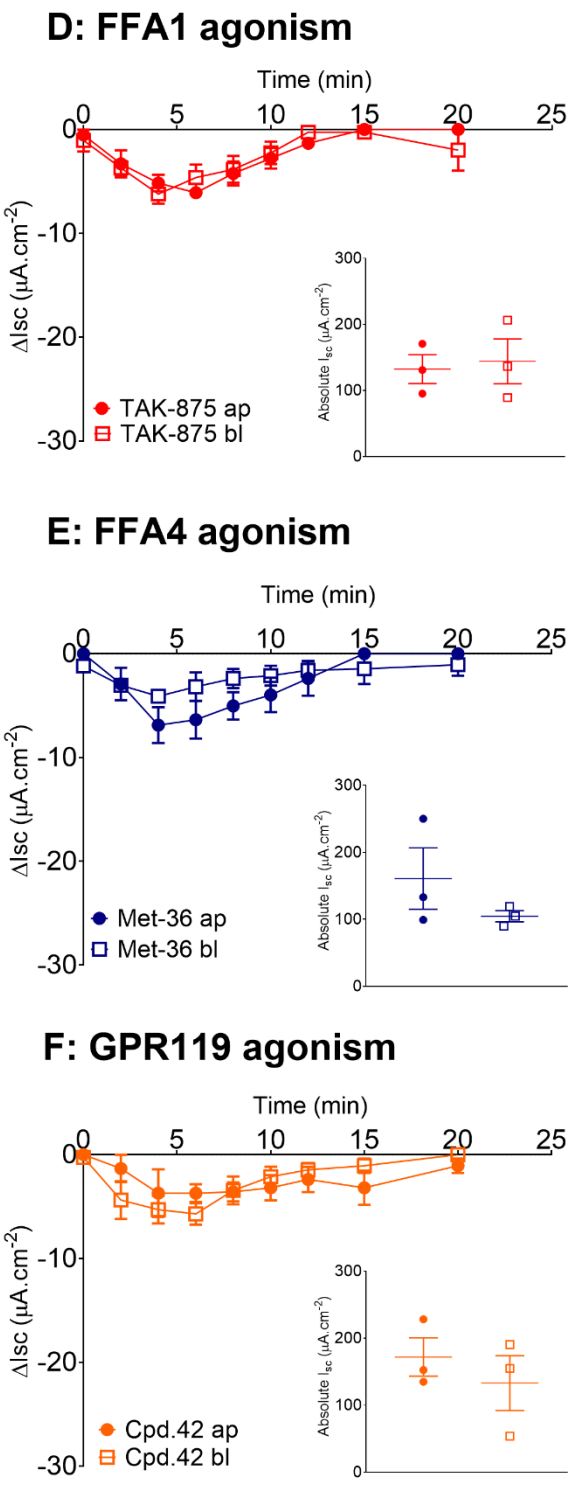


Figure 3.2 continued: Apical versus basolateral application of a FFA1, FFA4 and GPR119 agonist. In D: time courses of the anti-secretory response to the apical (ap) and basolateral (bl) applied FFA1 agonist, TAK-875 (200 nM, *n*=3); in E: the FFA4 agonist, Met-36 (100 nM *n*=3) and the GPR119 agonist, Cpd.42 in F (100 nM, *n*=3) after VIP pretreatment. The insets in D-F display absolute I_{sc} values at the point of

agonist administration. Symbols represent the number of observations and the coloured horizontal line displays the mean ± 1SEM. Time course points represent the mean ± 1SEM.

3.3 Regional variation of FFA1, FFA4 and GPR119 anti-secretory responses on basal I_{sc} and after VIP

Having established the EC_{50} values of the selective compounds, an EC_{80} concentration of the FFA1 agonist (TAK-875), the FFA4 agonist (Met-36) and the GPR119 agonist (Cpd.42) was selected to investigate the region GI variation of FFA1, FFA4 and GPR119 agonism. Agonism was assessed in the mid-ileum, the terminal ileum, ascending colon and descending colon. The change in I_{sc} was examined on basal I_{sc} and compared with the change in I_{sc} after VIP pretreatment.

3.3.1 FFA1, FFA4 and GPR119 agonism on basal I_{sc}

On basal I_{sc}, the TAK-875 anti-secretory response appeared larger in the ascending and descending colon compared with the terminal ileum, however this was not significant. The TAK-875 response in the descending colon was significantly larger than its respective DMSO control (Figure 3.4A).

On basal I_{sc}, the anti-secretory response of the FFA4 agonist, Met-36 was significantly larger in the ascending colon, compared with FFA4 agonism in the descending colon and terminal ileum. Additionally, FFA4 anti-secretory responses in the ascending and descending colon were significantly larger than their respective DMSO controls (Figure 3.4B).

The GPR119 anti-secretory responses on basal I_{sc} (Figure 3.4C) increased along the GI tract, with the descending colon providing the largest GPR119 response. The descending colon GPR119 anti-secretory response was significantly larger when compared with the response in the terminal ileum. Moreover, in the descending colon, the GPR119 anti-secretory response was significantly larger than its respective DMSO control.

3.3.2 FFA1, FFA4, GPR119 agonism after VIP

In mucosal preparations pretreated with VIP, lower concentrations of TAK-875, Met-36 and Cpd.42 (Figure 3.3D) induced anti-secretory responses that were more readily observed. TAK-875 responses were equally efficacious in the ascending colon, descending colon and terminal ileum. This pattern resembled TAK-875 agonism on basal I_{sc}. Furthermore, the response to TAK-875 in the mid-ileum was similarly efficacious to the responses observed in the ascending and descending colon. When comparing TAK-875 agonism after VIP with agonism on basal I_{sc}, evidently the responses after VIP were significantly larger in the terminal ileum and ascending colon.

In the presence of VIP, the area-specific responses of the FFA4 agonist, Met-36 were greatest in the ascending colon and slightly smaller in the descending colon (not significant), resembling a similar pattern to that observed on basal I_{sc}. The reduction in I_{sc} in the terminal ileum was similarly efficacious to the response observed in the ascending colon and significantly greater than that observed under basal conditions. Finally, the anti-secretory responses observed in the mid-ileum was similar to those seen in the descending colon (Figure 3.3D). There were no significant differences between the FFA1 and FFA4 responses in the presence of VIP, in each of the regions tested.

In the GI regions tested, the GPR119 Cpd.42 anti-secretory responses after VIP, resembled the same pattern as observed on basal I_{sc}. Furthermore, the descending colon mucosal Cpd.42 response was significantly larger than the response observed under basal conditions. In the presence of VIP, it was clear that the GPR119 responses observed in the descending colon were significantly larger than the responses observed in all other regions tested. This confirmed that GPR119 agonism (Cpd.42) incrementally increased along a gradient from the intestine to the colon, with the largest anti-secretory response observed in the colon, under basal conditions and after VIP.

In summary, FFA1, FFA4 and GPR119 anti-secretory responses were more readily observed after VIP compared with responses observed on basal I_{sc}, in various regions of the GI tract. The descending colon was selected as the optimal region for subsequent studies, as this area of the GI tract is highly enriched with enteroendocrine

PYY-containing L cells, which express the lipid metabolite receptors, FFA1 (Edfalk et al., 2008), FFA4 (Hirasawa et al., 2005) and GPR119 (Chu et al., 2008). Thus, the descending colon provided the greatest possibility of revealing specific L cell-mediated FFA1, FFA4 and GPR119 signalling mechanisms.

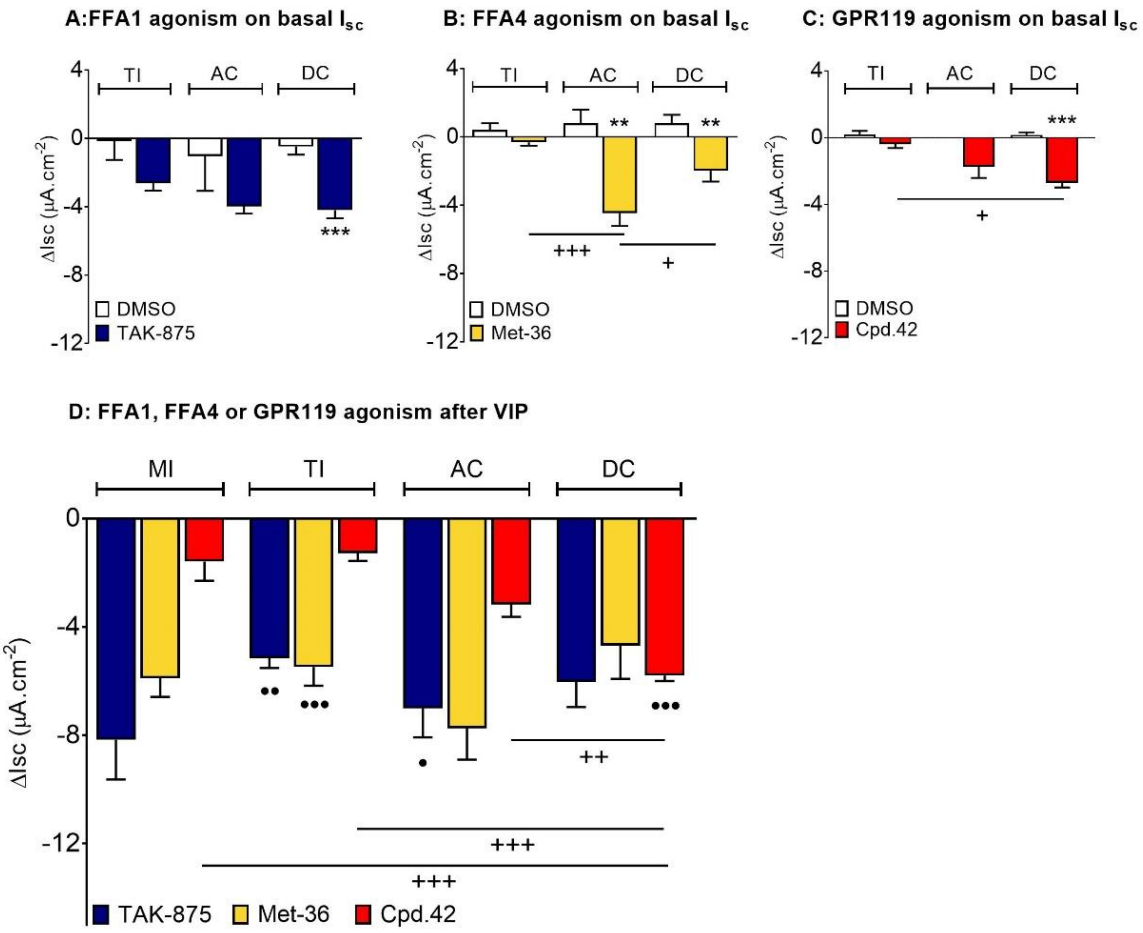


Figure 3.3: Regional variation of FFA1, FFA4 and GPR119 agonism on basal I_{sc} (A-C) and after VIP pretreatment (D). The responses to the FFA1 agonist, TAK-875 (1 μM, ap, *n*=5) (A), the FFA4 agonist, Met-36 (300 nM, *n*=5) (B) and the GPR119 agonist, Cpd.42 (300 nM, *n*=4-5) (C) on basal I_{sc} in the terminal ileum (TI), ascending colon (AC) and descending colon (DC), compared to 0.1 % DMSO treated controls. In D: the anti-secretory responses of the FFA1 agonist, TAK-875 (200 nM, ap), the FFA4 agonist, Met-36 (100 nM, ap) and the GPR119 agonist, Cpd.42 (100 nM, ap) after VIP pretreatment in the mid-ileum (MI), TI, AC and DC (*n*=4-7). Statistical differences from vehicle controls in each GI region on basal I_{sc} are represented as follows; ** $P \leq 0.01$ and ***$P \leq 0.001$ (Student's *t*-test). Differences

between anti-secretory responses on basal I_{sc} or after VIP between GI regions (MI, TI, AC and DC) are shown as follows; $^{+}P \leq 0.05$, $^{++}P \leq 0.01$ and $^{+++}P \leq 0.001$ (one-way ANOVA with Bonferroni's test). Differences between VIP pretreated agonist-induced anti-secretory responses (in D) and agonism under basal conditions (A-C) are shown as follows; $^{\bullet}P \leq 0.05$, $^{\bullet\bullet}P \leq 0.01$ and $^{\bullet\bullet\bullet}P \leq 0.001$ (Student's *t*-test). Bars represent the mean ± 1SEM.

3.4 FFA1, FFA4 and GPR119 anti-secretory responses were tetrodotoxin-insensitive

To determine whether the FFA1, FFA4 and GPR119 responses were neuronally-mediated, descending colon mucosal preparations were pretreated with the voltage-gated sodium channel neurotoxin, TTX. On basal I_{sc}, TTX significantly decreased I_{sc} in comparison to the vehicle (dH_2O), revealing blockade of endogenous enteric neuronal transmission (Figure 3.4A). The FFA1 (TAK-875), FFA4 (Met-36) and GPR119 (Cpd.42) anti-secretory responses were unaffected in the presence of TTX, compared to dH_2O-treated controls (Figure 3.4B). This indicated that the FFA1, FFA4 and GPR119 responses were most likely epithelial in origin and not neuronally-mediated. Likewise, the VIP secretory responses (Figure 3.4C) and the exogenous PYY responses were also TTX-insensitive (Figure 3.4D).

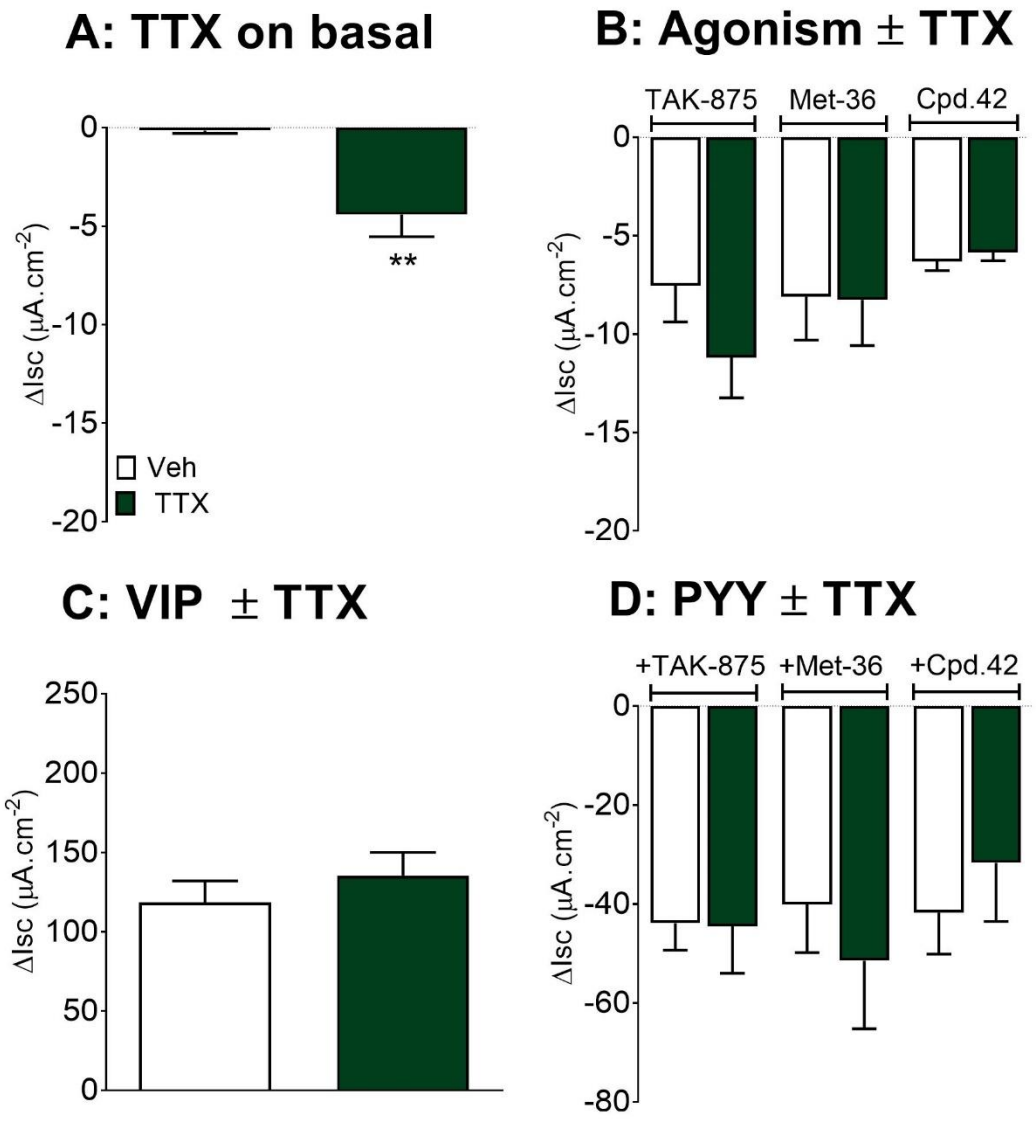


Figure 3.4: FFA1, FFA4 and GPR119 responses are TTX-insensitive. In A: basal changes in I_{sc} after administration of TTX (100 nM, bl) compared with vehicle control (Veh: dH_2O). In B: TAK-875 (200 nM, *n*=5), Met-36 (100 nM, *n*=5) and Cpd.42 (100 nM, *n*=10) responses after VIP pretreatment in the absence and presence of TTX (*n*=5). The vehicle controls, VIP (30 nM) and exogenous PYY (10 nM) are seen in C and D, respectively. The statistical difference between the pretreatments on basal I_{sc} is shown as follows; ** $P \leq 0.01$ (Student's *t*-test). Bars represent the mean ± 1SEM.

3.5 GLP-1 responses were region-specific in the colon and larger in the ascending colon

The GLP-1 agonist, Ex4, previously induced GLP-1-mediated secretory responses in the ascending colon. These responses were larger than the Ex4 response observed in the descending colon and were also Ex(9-39)-sensitive (Joshi et al., 2013). The variability in these region-specific responses was further investigated along the length of the mouse colon, by dividing the colon into eighths (~ 1 cm each). Each colon routinely provided 3 ascending colon mucosal preparations (AC1 – AC3), 2 transverse preparations: the ascending transverse colon (ATC), the transverse-descending colon (TDC) and 3 descending colon preparations (DC3 – DC1) (Figure 3.5A). Next, the response to Ex4 on basal I_{sc} was examined in the absence and presence of the GLP-1 antagonist, Ex(9-39), in the newly divided regions of the colon. In the absence of Ex(9-39), Ex4 provided an increasing secretory response gradient, from the descending region of the colon (DC1) towards the ascending region (AC1). In the descending colon regions, Ex4 responses were small and anti-secretory. Further along in the transverse colon, the Ex4 responses transitioned from an anti-secretory response into a secretory response. These secretory responses in the transverse colon appeared larger than the responses observed in the descending colonic areas, but this was not significant. The largest Ex4 response was observed in AC1 – AC2, followed by AC3-ATC. In all regions tested, the Ex4 response was abolished in the presence of Ex(9-39) and was therefore GLP-1R-mediated (Figure 3.5B). Thus, Ex4-induced GLP-1 responses in the mouse colon were region-specific, and the ascending colon (AC1 – AC2) was the optimal colonic area to use in further GLP-1 studies.

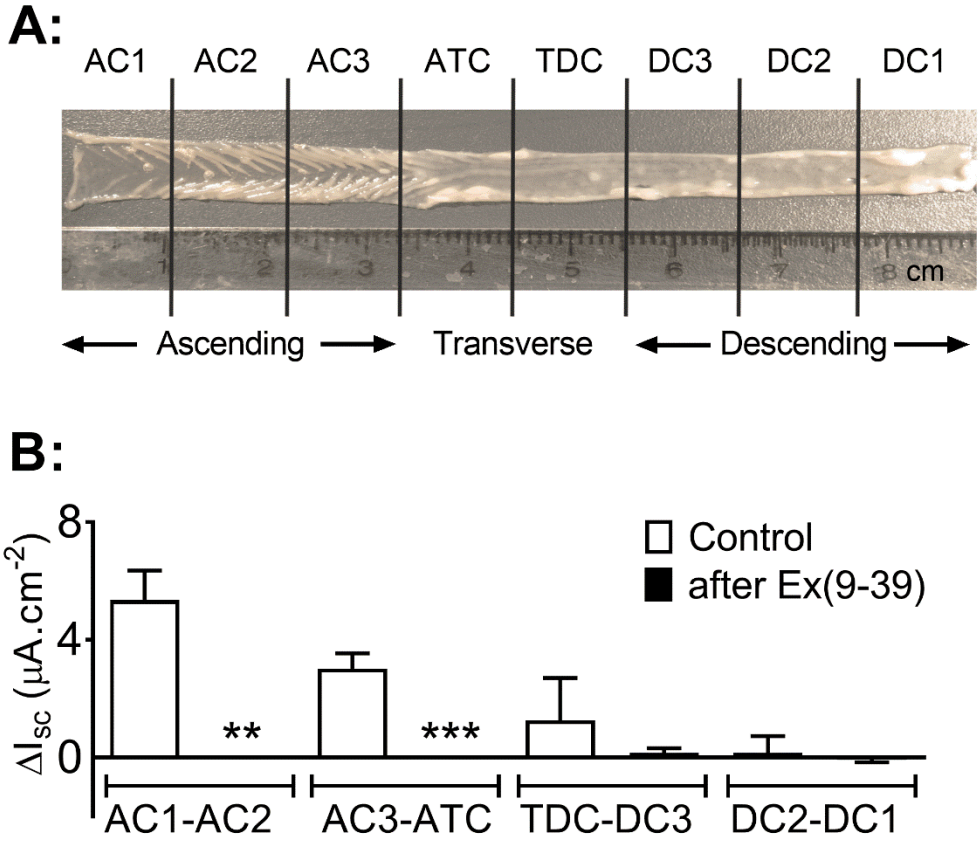


Figure 3.5: Region-specific GLP-1 responses in the mouse colon. In A: a photograph of the caeco-colorectal mucosal preparation (taken by IR Tough). Each colon was divided into eight mucosal preparations. The ascending colon provided AC1-AC3, the transverse was divided into the ascending-transverse colon (ATC) and the transverse-descending colon (TDC) and finally the descending colon provided three sections, DC3-DC1. In B: Ex4 (100 nM, bl) responses on basal I_{sc} in the absence (Control: dH_2O) and presence of Ex(9-39) (1 μM) along the length of the colon (n=5). Significant differences between Ex4 in the absence and presence of Ex(9-39) are shown as follows; **$P \leq 0.01$ and *** $P \leq 0.001$ (Student's t-test). Bars represent the mean ± 1SEM.

3.6 PSN632408 responses were insensitive to the DPPIV-inhibitor, sitagliptin

Earlier in this study, the GPR119 full agonist, PSN632408 induced a monophasic anti-secretory response in the descending colon (Figure 3.2C). However, no GLP-1 component was observed. Having established that the AC1-AC2 region provided the largest Ex4 GLP-1 response, the PSN632408 anti-secretory response was assessed in this region. This provided the optimal GI region to reveal a GLP-1 component to GPR119 agonism. The presence of the DPPIV inhibitor, sitagliptin ensured any GLP-1 response could potentially be stabilised and therefore elevated. For comparison, mucosa from the ascending colon (AC1 – AC2) was compared with descending colon mucosa (DC2- DC1). These two areas were incubated with an optimal concentration of sitagliptin or its respective vehicle control (dH_2O).

In the absence of sitagliptin, PSN632408 induced similarly efficacious monophasic reductions in I_{sc} in the ascending and descending regions of the colon. In the presence of sitagliptin, the PSN632408 responses were unaffected and remained monophasic in both regions of the colon, when compared with their respective vehicle controls (Figure 3.6A). In the absence of sitagliptin, Ex4 responses after PSN632408 were significantly larger in the ascending region when compared with the descending region (Figure 3.6B), which was consistent with the findings in the earlier GLP-1 region-specific study (Figure 3.5B). In the presence of sitagliptin, the Ex4 response in the ascending region exhibited an apparent effect, however this was not significant (P = 0.2) (Figure 3.6B). Moreover, the Ex4 response in the ascending colon was significantly larger than the Ex4 response in the descending region, in the presence of the DPPIV inhibitor. Sitagliptin had no effect on Ex4 responses in the descending colon. Exogenous PYY responses after the Ex4 additions were unaffected by the presence of sitagliptin, in both regions of the colon (Figure 3.6C).

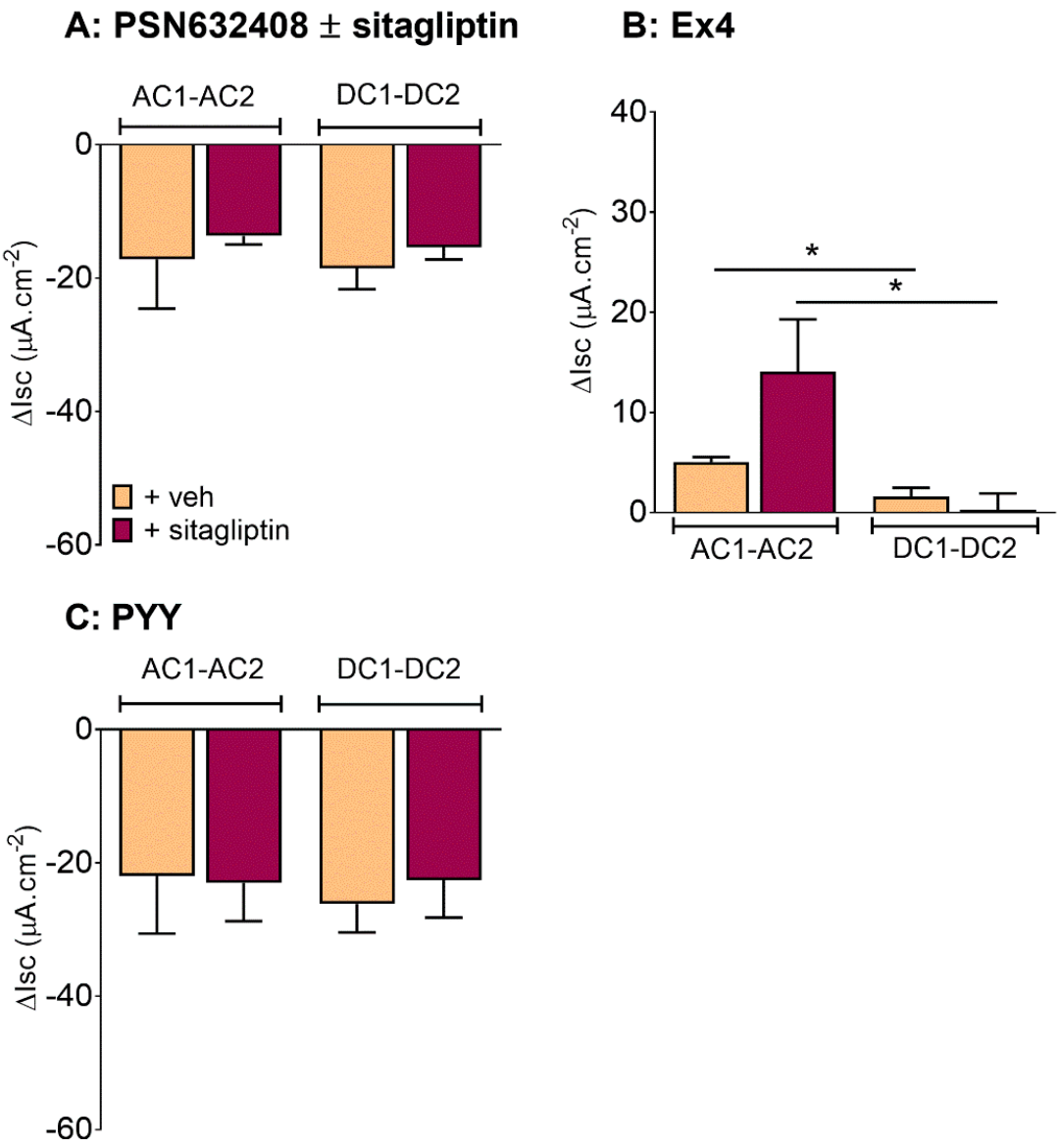


Figure 3.6: PSN632408 responses were insensitive to the DPPIV-inhibitor, sitagliptin. In A: the responses of the GPR119 full agonist, PSN632408 (10 μM, ap) after VIP pretreatment in the absence (+ veh: dH_2O) and presence of the DPPIV-inhibitor, sitagliptin (1 μM, bl) in the ascending colon (AC1-AC2) (*n*=3-4) and descending colon (DC1-DC2) (*n*=5-6). In B: regional Ex4 (100 nM, bl) responses after PSN632408 in the presence or absence of sitagliptin. In C: exogenous PYY (10 nM, bl) responses after Ex4 in the presence of sitagliptin or vehicle control. Statistical differences between Ex4 responses in the AC region and those in the DC region, in the absence and presence of sitagliptin are shown as follows, * $P \leq 0.05$ (Student's *t*-test). Bars represent the mean ± 1SEM.

3.7 GW1100 and ANT825 each inhibited agonist-induced FFA1 responses in the descending colon

The FFA1 antagonists, GW1100 and ANT825 alone revealed a similar degree of FFA1 tonic activity under basal conditions in the descending colon (Figure 3.7A). The FFA1 tonic activity of ANT825 was examined under basal conditions in the duodenum, mid-ileum, terminal ileum and ascending colon, revealing regional variation of FFA1 tone. The level of FFA1 tonic activity appeared uniform in all GI regions tested. Moreover, the tonic activity observed in the duodenum, mid-ileum, terminal ileum and ascending colon was similar to that seen in the descending colon (Figure 3.7B). ANT825 inhibited JTT (300 nM, ap) responses competitively, with an IC_{50} of 219 nM (Figure 3.7C). Optimal blocking concentrations of each FFA1 antagonist, GW1100 or ANT825, revealed the selectivity of the commercially available agonists, TUG424 and GW9508 for the FFA1 receptor. The GW9508 response was partially reduced in the presence of ANT825 or GW1100. Additionally, TUG891 responses were not affected by either FFA1 antagonists (Figure 3.7D). FFA1 agonism (utilising the AZ selective FFA1 agonists, TAK-875 and JTT) was also significantly inhibited by ANT825, showing FFA1 selectivity, whereas FFA4 (Met-36 and AZ423) and GPR119 (Cpd.42) agonism was unaffected (Figure 3.7E).

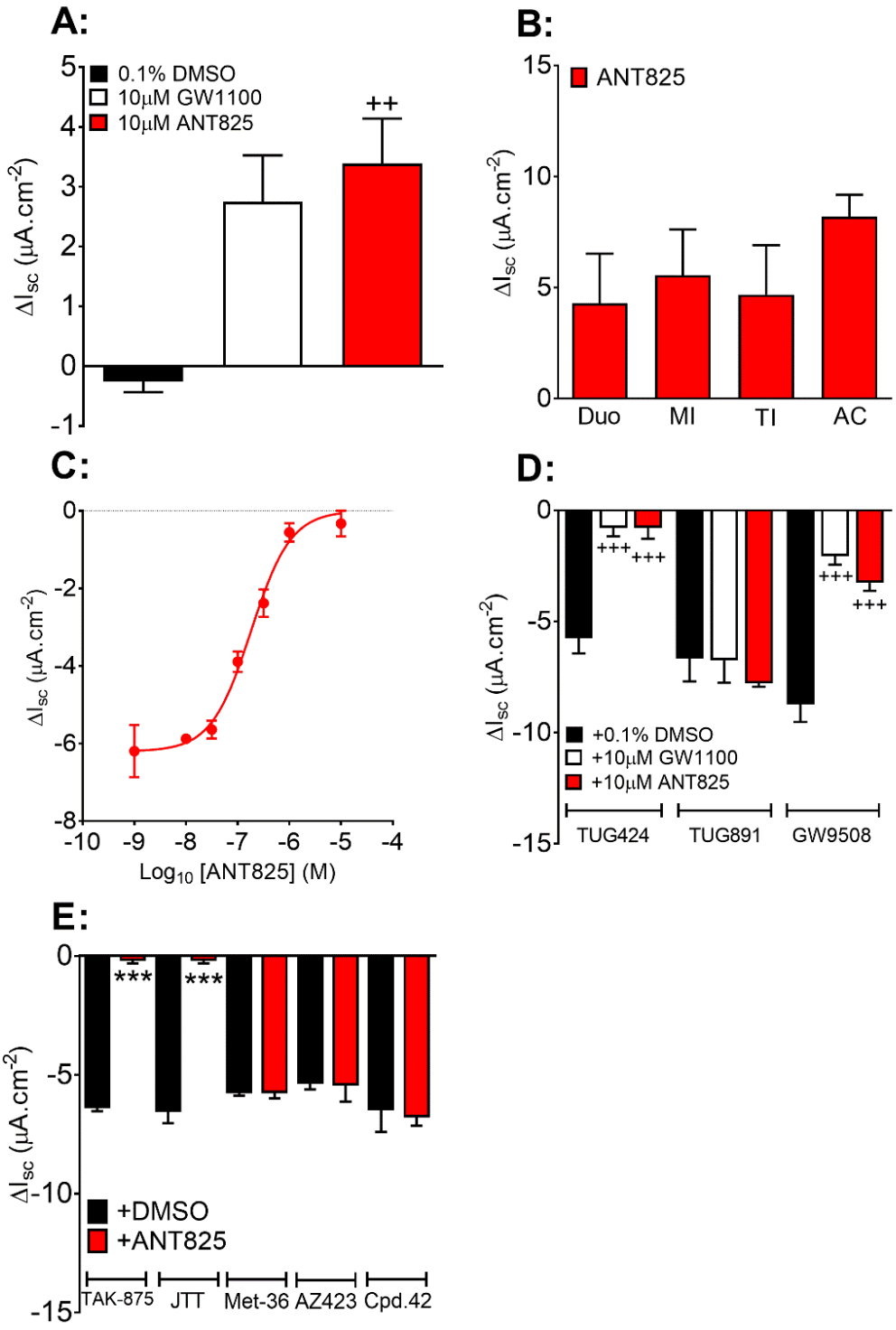


Figure 3.7: Tonic FFA1 activity and inhibition of FFA1 responses by GW1100 or ANT825. In A: changes in I_{sc} to vehicle (DMSO) or the FFA1 antagonists, GW1100 or ANT825 alone on basal I_{sc} in descending colon. In B: regional changes in I_{sc} to ANT825 (10 μM) in the duodenum (duo, *n*=5), MI (*n*=4), TI (*n*=5) and AC (*n*=5)

under basal conditions. In C: competitive inhibition of the FFA1 agonist, JTT responses (300 nM) after VIP, by pretreatment with the FFA1 antagonist, ANT825 in the descending colon. Each point is the mean ±1SEM (*n*=5-6). In D: pooled data shows the selective inhibition of apical TUG424 (100 nM, *n*=5-6) and GW9508 (1 µM, *n*=5) responses, but not TUG891 (100 nM, *n*=5) responses following apical GW1100 or ANT825 treatment compared with their respective vehicle controls (DMSO). In E: selective inhibition of the FFA1 agonists, TAK-875 (200 nM, *n*=5) and JTT (300 nM, *n*=5) responses but not the FFA4 agonist, AZ423 (100 nM, *n*=5) and Met-36 (100 nM, *n*=5) responses or GPR119, Cpd.42 (100 nM, *n*=4) responses following apical ANT825 treatment (10 µM, *n*=5). Bars represent the mean ± 1SEM. Statistical differences from respective vehicle controls are shown as follows; *** $P \leq 0.001$ (Student's *t*-test) and $^{++}P \leq 0.01$ and $^{+++}P \leq 0.001$ (one-way AVOVA with Dunnett's *post hoc* test).

3.8 AH-7614 inhibited agonist-induced FFA4 responses, not FFA1 responses in the descending colon

First, the FFA4 antagonist, AH-7614 inhibited Met-36 responses (Figure 3.8A) and provided an IC_{50} of 42.2 nM. Using a supramaximal blocking concentration of AH-7614, in combination with a previously optimised concentration of the FFA1 antagonist, ANT825, the selectivity of the dual FFA1 and FFA4 commercially available agonist, GW9508 was assessed. In the presence of both antagonists, the GW9508 response was significantly inhibited (Figure 3.8B). Additionally, this response was significantly different from the GW9508 response in the presence of ANT825 alone in Figure 3.7D (** $P \leq 0.01$, Student's *t*-test). This confirmed that GW9508 exerts dual FFA1 and FFA4 agonism. Furthermore, AH-7614 had no effect on the FFA1 TAK-875 response (Figure 3.8B). Under basal conditions, the FFA4 antagonist, AH-7614 did not affect the basal I_{sc} (data not shown). Furthermore, the previously observed FFA1 tone revealed in the presence of the FFA1 antagonist, ANT825 (Figure 3.7A) was eliminated when this antagonist was applied in combination with the FFA4 antagonist under basal conditions (AH-7614+ANT825; -1.23 ± 0.93 $\mu A.cm^{-2}$). This latter response was significantly different (** $P \leq 0.01$, Student's *t*-test) from the previously observed FFA1 tone (Figure 3.7A).

The basal I_{sc} changes to AH-7614, ANT825 or the combined antagonists, were assessed in the presence of pharmacological Y_1 and Y_2 blockade or vehicle (DMSO). In the presence of DMSO, AH-7614 had no effect on basal I_{sc}, as described previously. However, in the presence of the Y_1 and Y_2 antagonists, AH-7614 revealed an anti-secretory response and this was significantly different from the corresponding control (Figure 3.8C). ANT825 revealed FFA1 tonic activity as observed earlier (Figure 3.7A), and this response was inhibited in the presence of the combined Y_1 and Y_2 inhibitors. Thus, FFA1 tone was PYY Y_1/Y_2-mediated (Figure 3.8C). In the presence of vehicle, the combined FFA1 and FFA4 antagonists decreased the I_{sc}, and a significantly larger anti-secretory response was observed in the presence of the competitive Y_1/Y_2 antagonists (Figure 3.8C). Exogenous PYY responses were significantly inhibited in the presence of Y_1 and Y_2 receptor blockade, in comparison to vehicle-treated controls (data not shown).

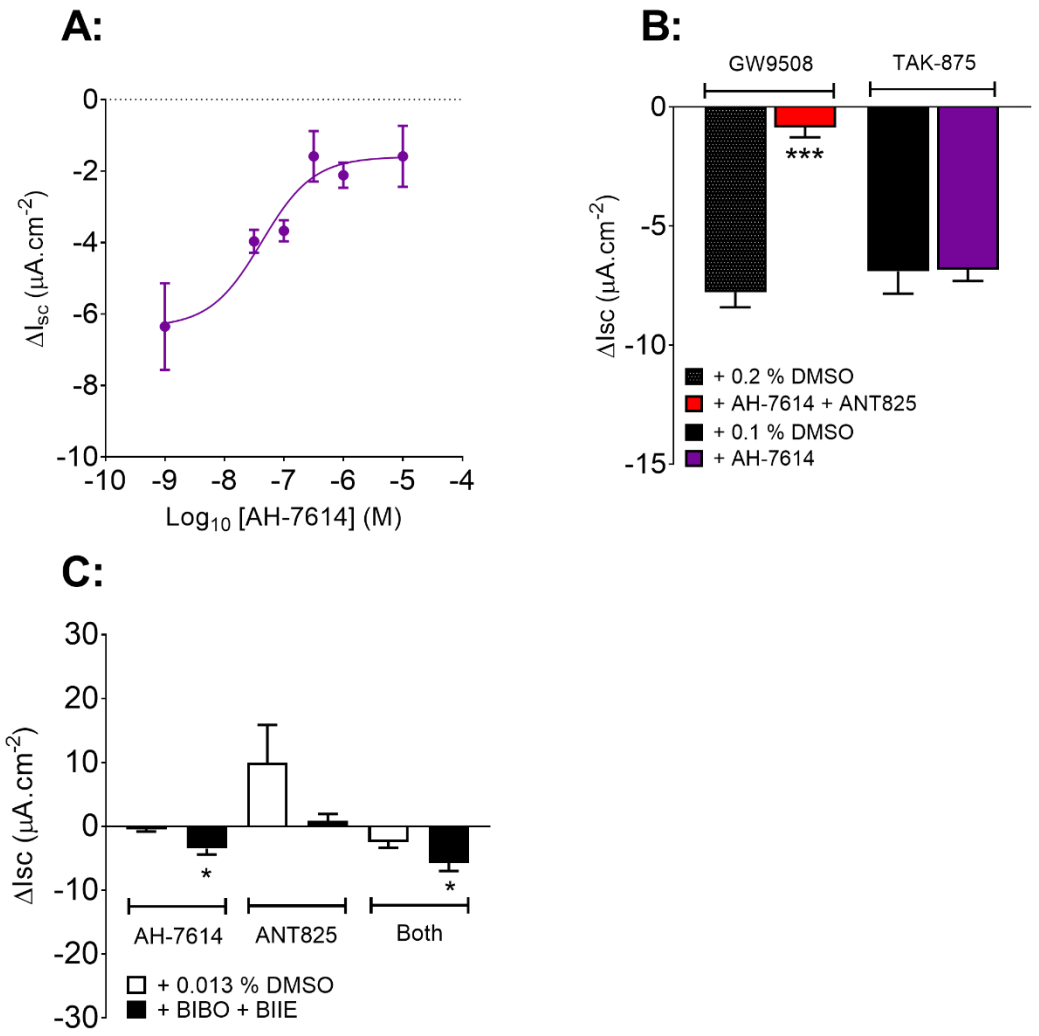


Figure 3.8: Inhibition of FFA4 responses by the FFA4 antagonist, AH-7614 in descending colon. In A: competitive inhibition of the FFA4 agonist, Met-36 responses (100 nM, after VIP) by pretreatment with the varying concentrations of the FFA4 antagonist, AH-7614. Each point is the mean ± 1SEM (*n*=3-4). In B: pooled data shows the selective inhibition of apical GW9508 (1 µM, *n*=5) but not TAK-875 responses (200 nM, *n*=5), following the apical FFA4 antagonist AH-7614 (10 µM) ± FFA1 antagonist ANT825 (10 µM), compared with respective vehicle controls (0.1 % DMSO and 0.2 % DMSO). In C: the effect of apical AH-7614 (10 µM, *n*=5), ANT825 (10 µM, *n*=5) and the combination of both antagonists (Both, *n*=5) on basal I_{sc}, in the presence of Y_1 (BIBO3304; BIBO (300 nM)) and Y_2 receptor blockade (BIIE2046; BIIE (1 µM)) or vehicle control (0.013 % DMSO). Bars/points represent the mean ± 1SEM. The statistical differences from respective vehicle controls are shown as follows; * $P \leq 0.05$ and *** $P \leq 0.001$ (Student's *t*-test).

3.9 FFA1, FFA4 and GPR119 colonic anti-secretory responses were Y_1-BIBO3304 sensitive but not Y_2-BIIE0246 sensitive

Endogenous PYY mediation of the FFA1 (JTT and TAK-875), FFA4 (AZ423 and Met-36) and GPR119 (Cpd.42) agonist responses were determined by pharmacological blockade of Y_1 or Y_2 receptors, utilising the Y_1 antagonist, BIBO3304; the Y_2 antagonist, BIIE0246 or a combination of both Y antagonists. Each antagonist revealed endogenous Y_1 and Y_2 tonic activity under basal conditions (Figure 3.9A), which was similar to that observed in the mouse colon previously (Hyland et al., 2003; Hyland and Cox, 2005; Tough et al., 2011). FFA1 responses to JTT (Figure 3.9B) and TAK-875 (Figure 3.9C) were abolished by the Y_1 antagonist, indicating PYY-Y_1 signalling predominantly mediates FFA1 responses. Subsequent exogenous PYY responses were also abolished by the Y_1, but not by the Y_2 antagonist alone, while the combination of the antagonists abolished PYY activity (data not shown). This confirmed exogenous PYY anti-secretory responses were also predominantly PYY Y_1-mediated. The responses to the FFA4 agonists, AZ423 (Figure 3.9D) and Met-36 (Figure 3.9E), and the GPR119 agonist, Cpd.42 (Figure 3.9F) were also abolished by the Y_1 but not the Y_2 antagonist. Thus FFA1, FFA4 and GPR119 responses in the mouse descending colon were predominantly Y_1 receptor-mediated.

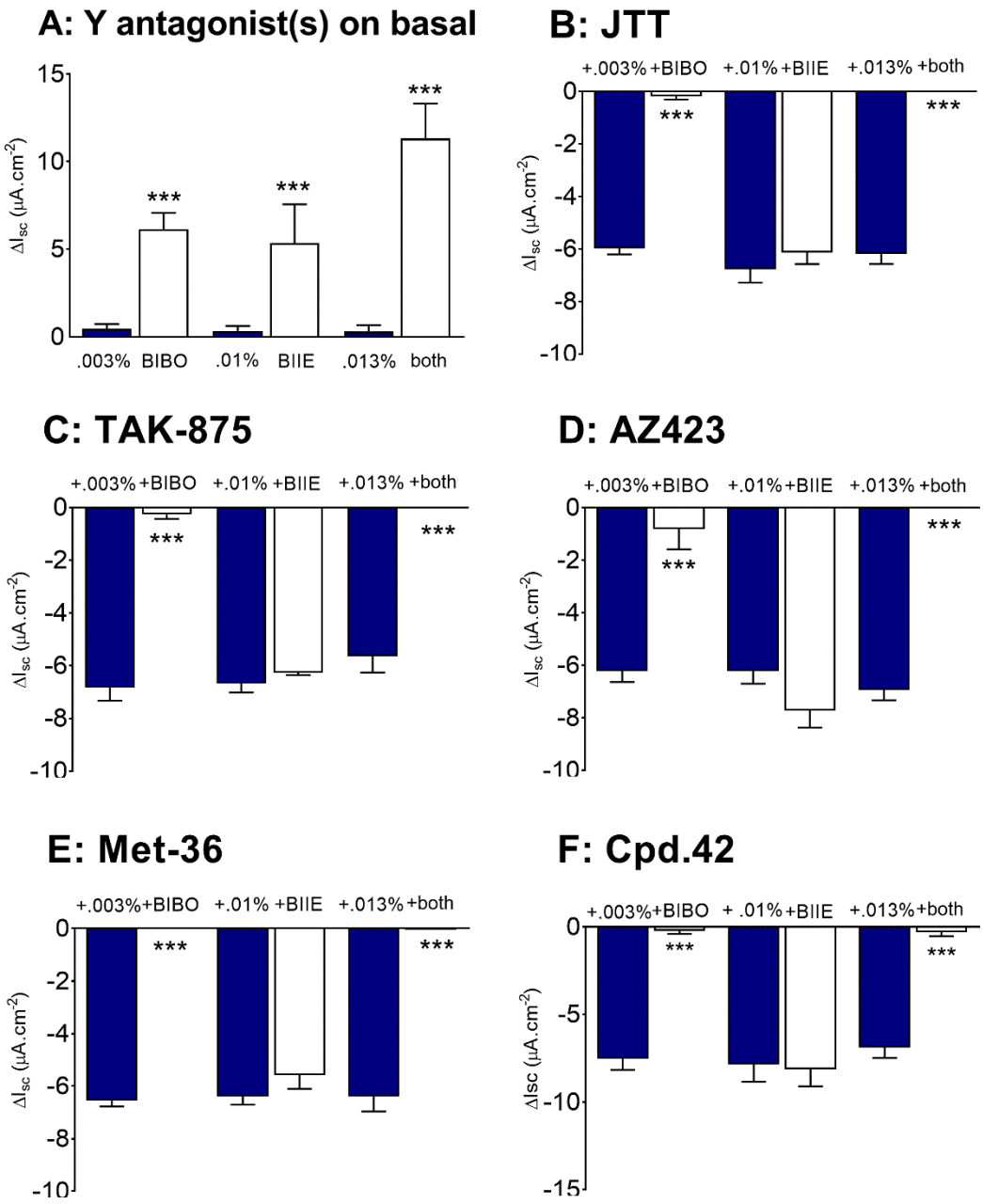


Figure 3.9: Y_1- but not Y_2-receptor sensitivity of FFA1, FFA4 and GPR119 agonism in the descending colon. The effects of the Y_1 (BIBO3304, BIBO, 300 nM) ± the Y_2 (BIIE0246, BIIE, 1 µM) antagonist and corresponding DMSO controls (+.003 %, +.01 %, +.013 %) on baseline I_{sc} levels are shown in A. The effect of the FFA1 agonists, JTT (300 nM, *n*=5-6) and TAK-875 (200 nM, *n*=5-6) after VIP in the absence or presence of Y antagonists are shown in B and C, respectively. The activity of the FFA4 agonists, AZ423 (100 nM, *n*=5) and Met-36 (100 nM, *n*=5-6) after VIP is shown in D and E respectively and the GPR119 agonist, Cpd.42 responses (100 nM, *n*=3-5)

is shown in F. Bars represent the mean ± 1SEM. Statistical differences between vehicle controls (0.003 %, 0.01 % and 0.013 % DMSO) and respective antagonists BIBO, BIIE or both are shown as follows; *** $P \leq 0.001$ (Student's *t* test).

3.10 Absolute I_{sc} levels at the point of TAK-875 or JTT addition were no different in the presence of vehicle or BIIE2046-treated preparations

Cox *et al.* (1988) demonstrated that the decrease in I_{sc} was dependent and influenced by the preceding level of secretory I_{sc} (Cox et al., 1988). As the FFA1 (TAK-875 and JTT) responses in the presence of the Y_2 antagonist (Figure 3.9B-C) were slightly reduced, this was suggestive of a minor role for Y_2 receptors, as seen previously (Tough et al. 2011). Hence, the absolute I_{sc} levels at the point of agonist addition were analysed to determine whether the subsequent agonist response was dependent on the residual $I_{sc.}$ At the exact point of adding the FFA1 agonist (TAK-875 (Figure 3.10A) or JTT (Figure 3.10B)), there were no differences between descending colon mucosal preparations incubated with vehicle or those treated with the Y_2 antagonist. Taken together, these observations confirm selective Y_1-mediation of FFA1 responses.

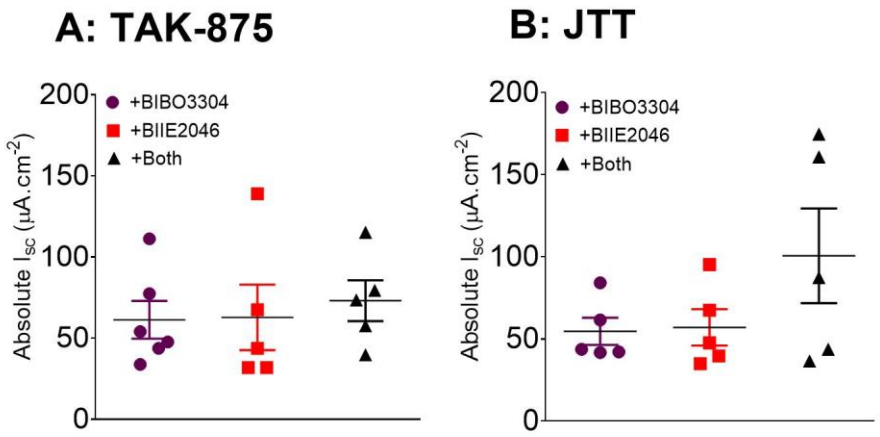


Figure 3.10: Absolute I_{sc} levels at the point of TAK-875 or JTT addition. The scatter plot shows the absolute I_{sc} values at the exact point of adding the FFA1 agonist(s), TAK-875 (A) or JTT (B), in the presence of the competitive Y_1 antagonist, BIBO3304 (300 nM), or the Y_2 antagonist, BIIE2046 (1 µM) or both Y antagonists. Symbols represents the number of observations and the black horizontal line displays the mean ± 1SEM.

3.11 FFA1, FFA4 and GPR119 responses were glucose-sensitive in the descending colon mucosa

In order to establish the glucose-sensitivity of FFA signalling in descending colon mucosal preparations, the responses to the FFA1, FFA4 and GPR119 agonists were compared in the presence or absence of apical glucose (11.1 mM). Mannitol replacement of apical glucose abolished the FFA1 responses (Figure 3.11A-B) and inhibited FFA4 and GPR119 activity (Figure 3.11C-E) compared to vehicle. The internal control, phloridzin (added ap) decreased the I_{sc}, but as expected only in the presence of glucose (because apical SGLT1 requires glucose to function). Thus, FFA1, FFA4 and GPR119 receptors were activated in a glucose-sensitive manner. In contrast, the PYY anti-secretory responses, after the various agonists, were glucose-insensitive (Figure 3.11A-E). The exogenous PYY response after the GPR119 agonist, Cpd.42, in the presence of mannitol was significantly larger compared with the PYY response in the presence of glucose. The absolute I_{sc} level at the point of exogenous PYY addition was greater in these mannitol-bathed mucosal preparations as Cpd.42 was unable to lower the I_{sc}, and this was attributed to the lack of glucose.

In this way, the greater I_{sc} in these preparations, provided a larger residual I_{sc} for exogenous PYY to inhibit (Figure 3.11E).

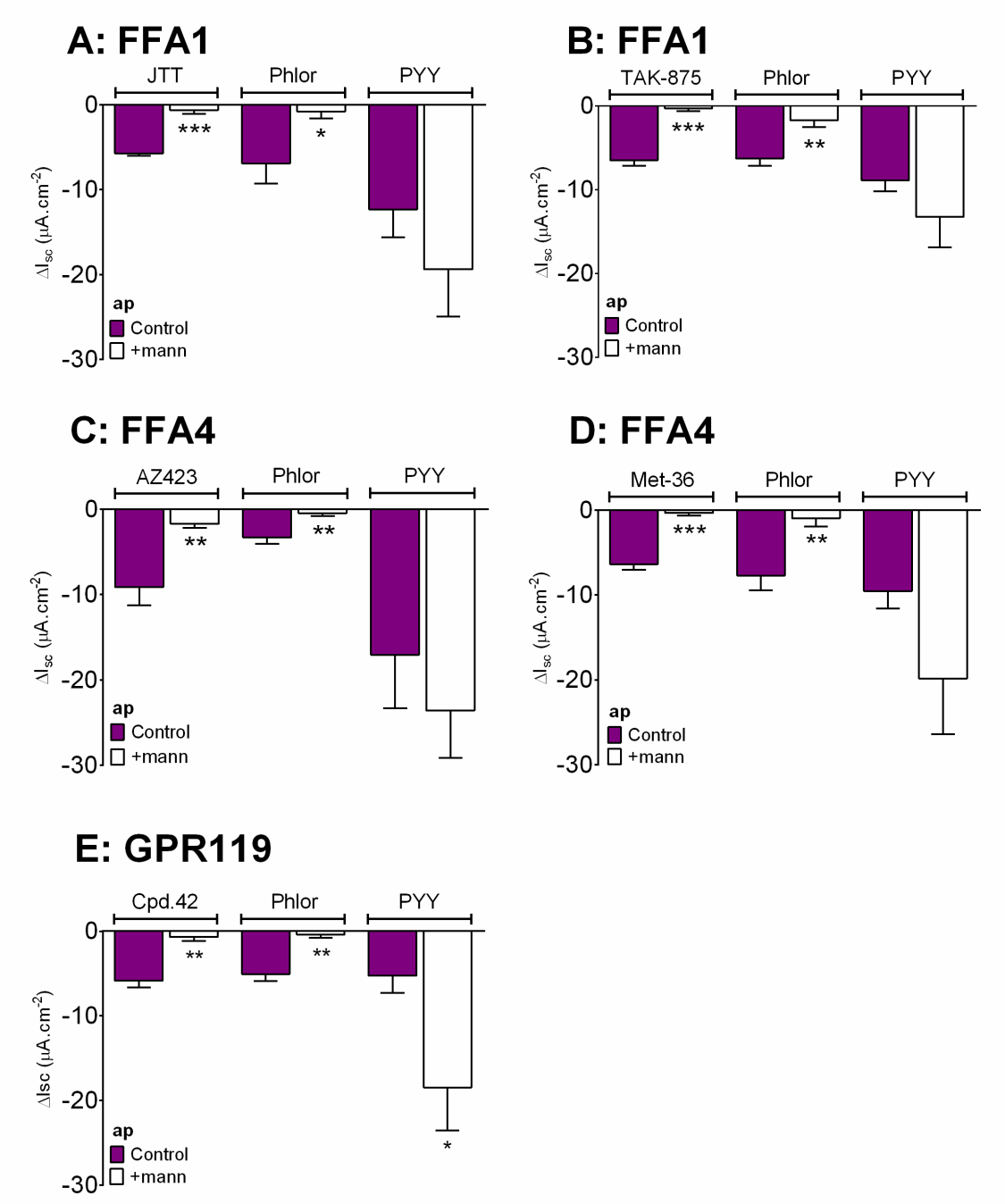


Figure 3.11: Glucose-sensitivity of FFA1, FFA4 and GPR119 agonism after VIP pretreatment in the descending colon. Glucose-sensitivity of apical FFA1 agonists, JTT (300 nM, *n*=5) in A, TAK-875 (200 nM, *n*=5) in B, apical FFA4 agonists, AZ423 (100 nM, *n*=5) in C and Met-36 (100 nM, *n*=5) in D and the apical GPR119 agonist Cpd.42 (100 nM, *n*=4) in E, in the presence and absence of 11.1 mM glucose. Control

mucosal preparations were bathed in glucose both sides whereas, mannitol (+ mann, 11.1 mM) replaced glucose apically only. Phloridzin (Phlor, 50µM, ap only) and PYY (10 nM) responses are also shown. Bars represent the mean - 1SEM. Statistical differences between agonist or phloridzin responses in the presence or absence of glucose, are shown as follows; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ (Student's *t*-test).

3.12 Blockade of SGLT1 and GLUT2 significantly decreased FFA1, FFA4 and GPR119 responses

To confirm the glucose-sensitivity of FFA1, FFA4 and GPR119 responses in descending colon mucosal preparations, mucosae were bathed in glucose on both sides of the reservoir and selective agonism was assessed in the presence and absence of the SGLT1 inhibitor, phloridzin and the GLUT2 inhibitor, phloretin. Firstly, under basal conditions the combination of the SGLT1 inhibitor, phloridzin and the GLUT2 inhibitor, phloretin caused a biphasic change in I_{sc}. The first initial decrease in I_{sc} was observed within the first 1-2 min, and the subsequent increase in I_{sc} was recorded within 10 min, indicative of SGLT1 and GLUT2 activity, respectively. The second component of the biphasic response was significantly different to the vehicle control (dh_2O) (Figure 3.12A). Next, the responses to the FFA1 agonist, TAK-875; the FFA4 agonist, Met-36 and the GPR119 agonist, Cpd.42 were assessed in the presence of both the SGLT1 and GLUT2 inhibitors. All three agonist responses were significantly attenuated in the presence of the inhibitors, in comparison to their respective vehicle controls (Figure 3.12B). Exogenous PYY responses after agonist addition were unaffected by SGLT1 and GLUT2 blockade (Figure 3.12C).

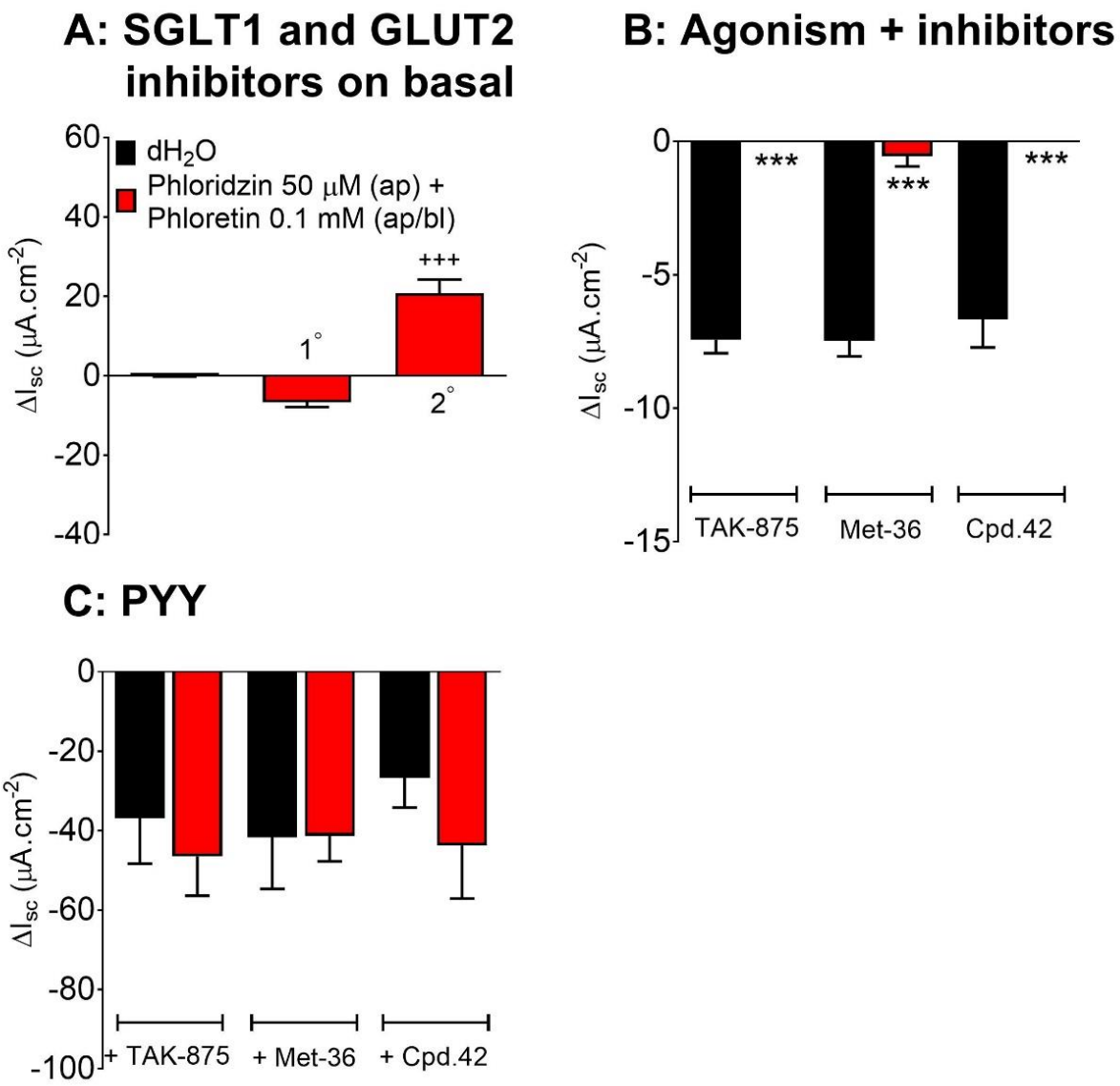


Figure 3.12: SGLT1 and GLUT2 inhibition significantly decreases FFA1, FFA4 and GPR119 anti-secretory responses. In A: the combination of the SGLT1 inhibitor, phloridzin (50 μM, ap) and the GLUT2 inhibitor, phloretin (0.1 mM, ap + bl) (*n*=5) or vehicle control, dh_2O (*n*=5) under basal conditions. The first initial decrease in I_{sc} upon application of Phloridzin + Phloretin was observed within the first 1-2 min, and the subsequent increase in I_{sc} was recorded within 10 min. In B: anti-secretory responses of TAK-875 (200 nM, ap), Met-36 (100 nM, ap) and Cpd.42 (100 nM, ap) after VIP, in the absence and presence of the inhibitors compared with respective vehicle controls (*n*=5). In C: PYY (10 nM, bl) responses after agonist administration in the presence and absence of the inhibitors (*n*=5). Statistical significant differences between responses in the presence of the SGLT1 + GLUT2

inhibitors and vehicle-treated controls are shown as follows *** $P \leq 0.001$ (Student's *t*-test). The difference between the pretreatments on basal I_{sc} is shown as follows; $^{+++}$ $P \leq 0.001$ (one-way ANOVA with Dunnett's *post hoc* test). Bars represent the mean ± 1SEM.

3.13 Co-agonism of FFA1, FFA4 or GPR119 was additive in the descending colon

To study L cell receptor co-agonism of FFA1, FFA4 or GPR119 in descending colon mucosal preparations, an EC_{80} concentration of each selective agonist was chosen. A single agonist was added to the apical reservoir and its response was monitored for 20 min and evaluated alone, or in combination with a second selective agonist. To study co-agonism, a combination of two selective agonists (added at the same time-point) were added apically, to a single colonic mucosal preparation. The time-courses of co-agonism versus single agonism were assessed for potential synergy.

3.13.1 FFA1 and FFA4 co-agonism

The time-course of TAK-875 (Figure 3.13A) shows that this agonist decreased the I_{sc} and its maximal response was recorded within 6 min, followed by a slow return of the I_{sc} to baseline. The time-course of Met-36 (Figure 3.13A) demonstrated that this FFA4 agonist induced an anti-secretory response that reached its maxima within 4 min, 2 min faster than the FFA1 agonist, TAK-875. The time-course of the combined agonists (TAK-875 + Met-36) indicated that together their responses were additive, and reached a maximal response at a slower pace of 6-8 min. The maximal response of the combined agonists (-13.9 ± 2.4 $\mu A.cm^{-2}$) was significantly different from the maximal TAK-875 response (-6.2 ± 0.7 $\mu A.cm^{-2}$, ** $P \leq 0.01$) and Met-36 response (-6.1 ± 0.9 $\mu A.cm^{-2}$, * $P \leq 0.05$), clearly showing co-agonism of FFA1 and FFA4 responses was additive. The time-course of the TAK-875 response was significantly different from the combined response at 5, 6 and 8 min. In contrast, the time-course of the Met-36 response was significantly different from the combined response at 5, 6, 8, and 10 min. The inset displays the absolute I_{sc} values at the point of agonist addition, indicating the absolute I_{sc} levels were no different between the three cohorts (Figure 3.13A inset).

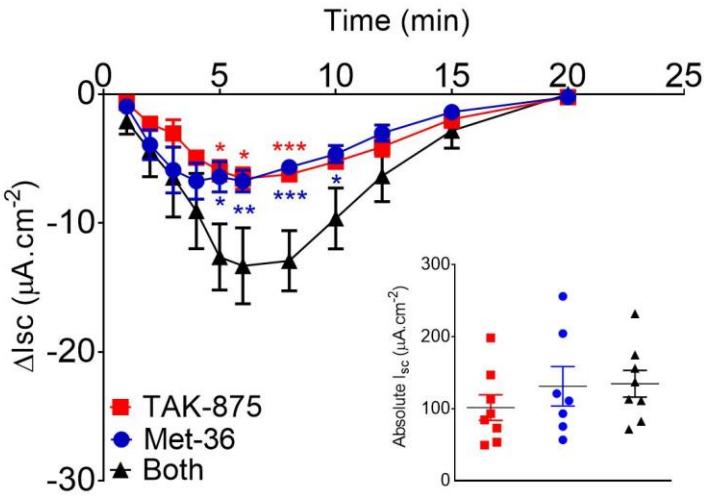
3.13.2 GPR119 and FFA1 co-agonism

The GPR119 Cpd.16 anti-secretory response in earlier experiments appeared partial, compared to the PSN632408 response (Figure 3.2C). Here, Cpd.16 decreased the I_{sc} and its maximal response was observed within 6 min. The time-course of the combined agonists (Cpd.16 + TAK-875) showed that co-agonism was additive and the maximal response was observed within 8-10 min (Figure 3.13B). This duration was 2-4 min longer than the maximal response observed for FFA1 and FFA4 co-agonism. The maxima of the combined agonist response (-12.2 ± 1.9 $\mu A.cm^{-2}$) was significantly different from the maximal TAK-875 response (-6.2 ± 0.5 $\mu A.cm^{-2}$, ** $P \leq 0.01$), but not the maximal Cpd.16 response (-7.5 ± 0.5 $\mu A.cm^{-2}$). The time-course of the TAK-875 response was significantly different from the combined response at 10, 12, 15 and 20 min. In contrast, the time-course of the Cpd.16 response was significantly different from the combined response at 8, 10, 12, 15 and 20 min. Overall, the combined TAK-875 + Cpd.16 response appeared additive. The inset shows that there were no differences between the absolute I_{sc} levels at the point of addition of TAK-875, Cpd.16 or the combined agonists (Figure 3.13B inset).

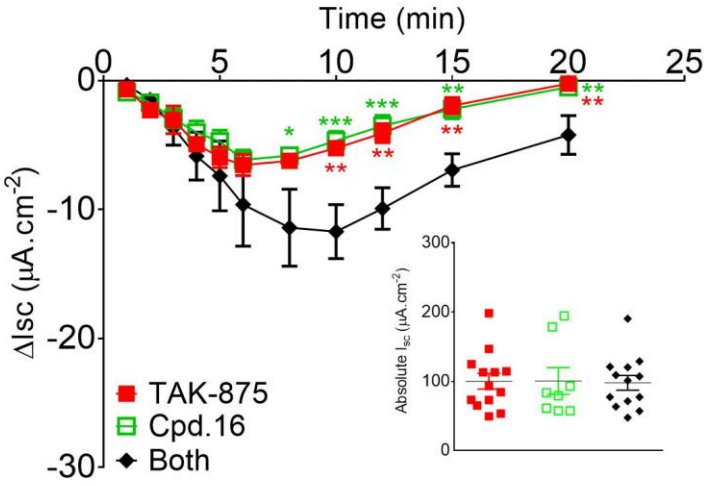
3.13.3 GPR119 and FFA4 co-agonism

The combined response to the FFA4 agonist and the GPR119 agonist, Cpd.16 was additive, and reached maximum within 7 min (Figure 3.13C), a duration similar to that of FFA1 and FFA4 co-agonism. The maximal combined response of Cpd.16 and Met-36 (-18.8 ± 2.5 $\mu A.cm^{-2}$) was significantly different from the maximal response of Cpd.16 GPR119 agonism (-7.3 ± 0.8 $\mu A.cm^{-2}$, *** $P \leq 0.001$) and Met-36 FFA4 agonism (-8.8 ± 1.2 $\mu A.cm^{-2}$, ** $P \leq 0.01$), indicating that together the Cpd.16 and Met-36 responses were additive. The time-course of the Met-36 response was significantly different from the combined response at 6, 8, 10, 12 and 15 min. In contrast, the time-course of the Cpd.16 response was significantly different from the combined response at 5, 6, 8, 10, 12 and 15 min. The inset shows there were no differences between absolute I_{sc} levels at the point of addition of Met-36, Cpd.16 or the combined agonists (Figure 3.13C inset).

A: FFA1 + FFA4 agonism



B: FFA1 + GPR119 agonism



C: FFA4 + GPR119 agonism

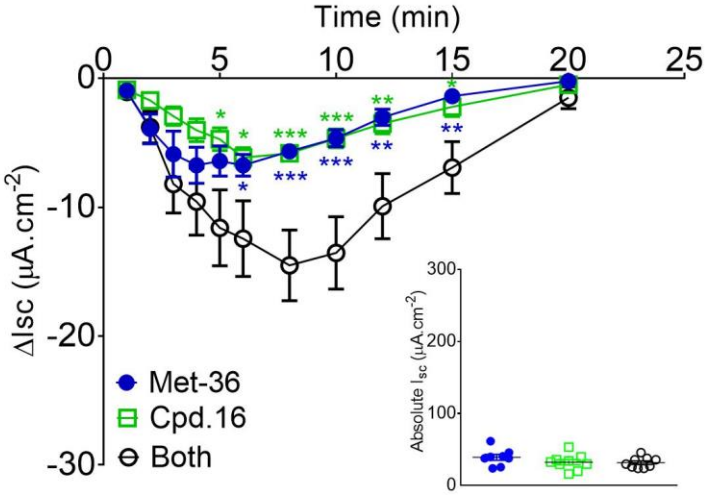


Figure 3.13: Co-agonism of FFA1, FFA4 or GPR119 after VIP pretreatment is additive. In A: time courses of the anti-secretory response to the FFA1 agonist, TAK-875 (1 μM, *n*=9); the FFA4 agonist, Met-36 (100 nM *n*=9) and the combination of TAK-875 + Met-36 (Both) (*n*=5). The insets in A-C display absolute I_{sc} values at the point of agonist administration. Symbols represent the number of observations and the black horizontal line displays the mean ± 1SEM. In B: time courses of anti-secretory response to the FFA1 agonist, TAK-875 (*n*=9); the GPR119 agonist, Cpd.16 (1 μM, *n*=9-10) and the combination of TAK-875 + Cpd.16 (Both) (*n*=5). In C: time courses of the anti-secretory response to the FFA4 agonist, Met-36; the GPR119 agonist, Cpd.16 and the combination of Met-36 + Cpd.16 (Both) (*n*=5). Notably, the time-course of individual agonist responses (TAK-875, Met-36 and Cpd.16) were duplicated in A-C to show the comparison between it, the second agonist response and their combined response. Significant differences between individual agonist responses and the combined agonists response were analysed using a one-way ANOVA, with Bonferroni's *post hoc* test. Statistical differences between each individual agonist response (at specific time-points) and the combined agonist response are shown as follows; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. Points represent the mean ± 1SEM.

3.14 Pre-stimulation of descending colon with the GPR119 agonist, PSN632408 inhibited subsequent GPR119 agonism but not subsequent FFA1 or FFA4 agonism

Descending colon mucosal preparations were incubated for 2 min with a high concentration of the commercially available GPR119 agonist, PSN632408 or its vehicle control, 95 % ethanol. Following the incubation period, the anti-secretory responses to the FFA1 agonist, TAK-875; the FFA4 agonist, Met-36 and the GPR119 agonists, PSN632408 or Cpd.42 were assessed in the presence or absence of PSN632408 pretreatment. In the presence of vehicle, each FFA1, FFA4 or GPR119 agonist decreased the I_{sc}. In the presence of PSN632408, the TAK-875 and Met-36 anti-secretory responses were no different to their corresponding controls, whereas the PSN632408 and Cpd.42 responses were significantly inhibited (Figure 3.14A). In the presence of PSN632408 pretreatment, exogenous PYY responses were not affected (Figure 3.14B). In summary, pre-stimulation of GPR119 reduces the response to subsequent GPR119 agonism but does not affect subsequent FFA1 or FFA4 agonism.

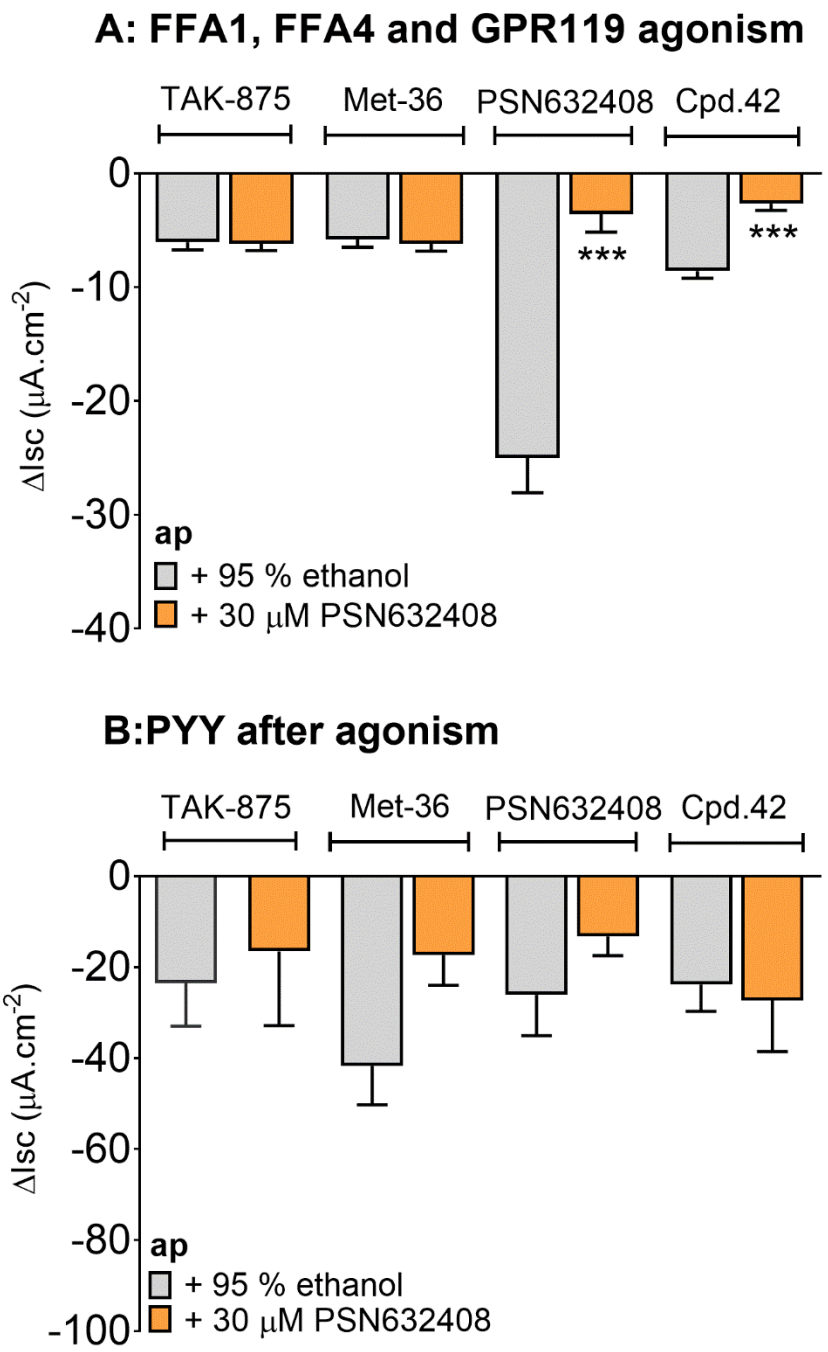


Figure 3.14: Apical pre-stimulation with a GPR119 agonist inhibits subsequent GPR119 agonism. In A: the anti-secretory responses of TAK-875 (200 nM, *n*=5), Met-36 (100 nM, *n*=4-5), PSN632408 (30 μM, *n*=5) and Cpd.42 (100 nM, *n*=5-6) after VIP pretreatment in the absence (95 % ethanol) and presence of PSN632408 (30 μM). In B: PYY (10 nM, bl) responses after agonist addition in the absence and presence of the pretreatments. The statistical difference between agonist responses in the presence

of PSN632408-pretreated or vehicle-treated controls (in A) are shown as follows, ***$P \leq 0.001$ (Student's *t*-test). Bars represent the mean - 1SEM.

3.15 Pinolenic acid was a dual FFA1 and FFA4 agonist and its response was PYY Y_1/Y_2-mediated in descending colon mucosa

The dietary metabolite, pinolenic acid has been reported as a dual FFA1 and FFA4 agonist. Here, pinolenic acid induced monophasic reductions in $I_{sc,}$ which were concentration-dependent, and provided an EC_{50} value of 298.2 nM (Figure 3.15A). Additionally, pinolenic acid was equipotent and as efficacious as the commercially available dual FFA1 and FFA4 agonist, GW9508 (seen previously in Figure 3.2A).

To establish the pharmacology of pinolenic acid in the descending colon, the mucosa was pretreated with the neuronal inhibitor, TTX; the FFA1 antagonist, ANT825; a combination of the FFA4 antagonist (AH-7614) + FFA1 antagonist (ANT825) or a combination of the Y_1 and Y_2 antagonists, BIBO3304 + BIIE2046, respectively. As shown earlier, under basal conditions TTX significantly decreased $I_{sc,}$ displaying loss of endogenous neuronal activity, ANT825 significantly increased I_{sc} revealing FFA1-tonic activity, the combination of the FFA1 and FFA4 antagonists slightly decreased basal I_{sc} but this was not significant, and the combination of Y_1 and Y_2 antagonists increased I_{sc} displaying Y_1 and Y_2 tone (Figure 3.15B). In the presence of these various pretreatments, the pinolenic acid anti-secretory response was TTX-insensitive, partially inhibited by ANT825 (as seen previously with GW9508), significantly inhibited in the presence of both FFA1 and FFA4 antagonists, and its activity was abolished by the Y_1+Y_2 antagonists (Figure 3.15C). Furthermore, the pinolenic response in the presence of both FFA1 and FFA4 antagonists was significantly different from the response in the presence of the FFA1 antagonist, ANT825 alone (Figure 3.15C). This indicated that pinolenic acid selectively activated FFA1 and FFA4 receptors in the descending colon, and its response was epithelial in origin and Y_1/Y_2-mediated. As expected, exogenous PYY responses after Y_1 and Y_2 blockade were abolished compared to the vehicle control, whereas PYY responses in the presence of all other pretreatments were unaffected (Figure 3.15D).

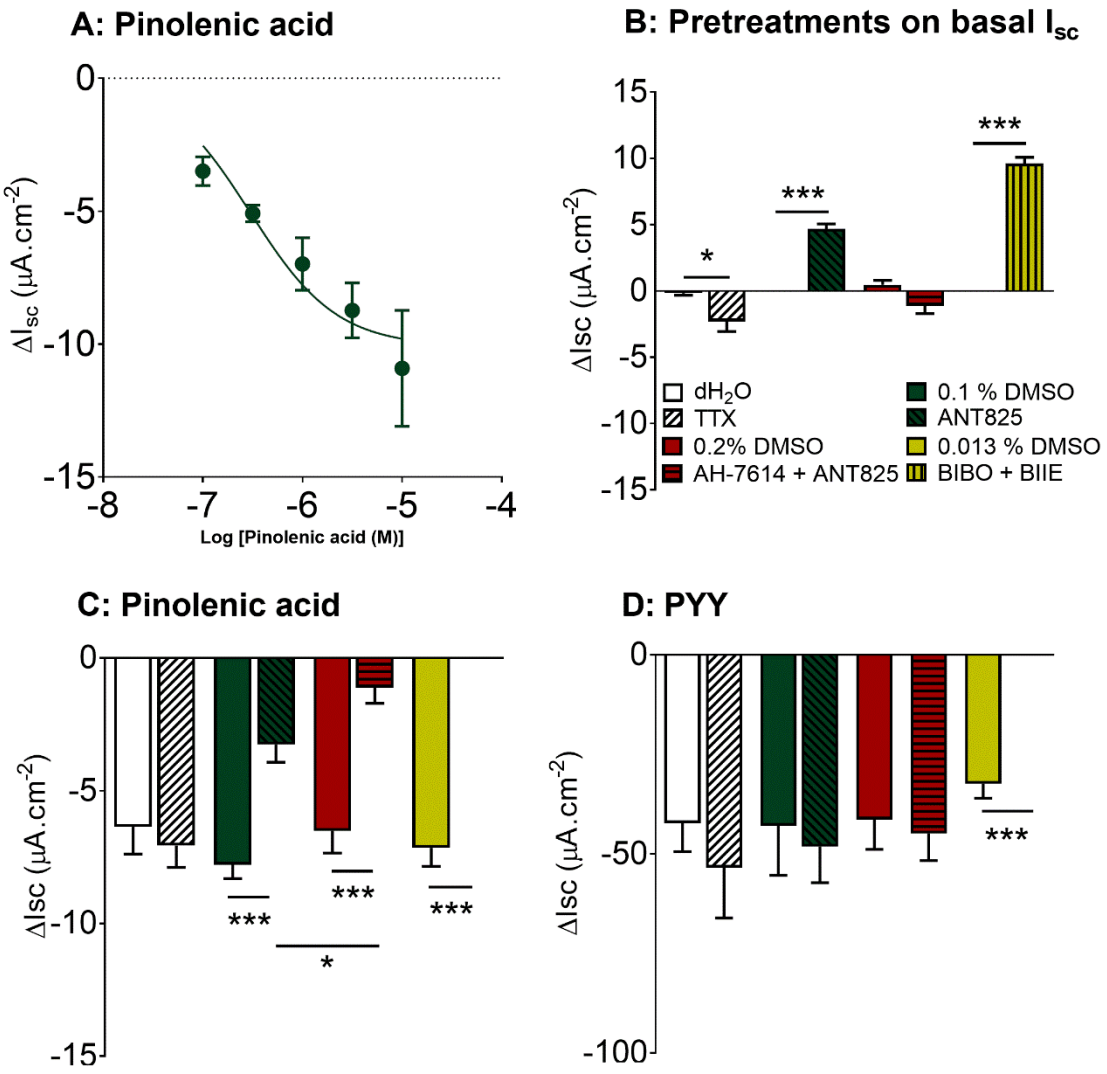


Figure 3.15: The anti-secretory response to pinolenic acid is FFA1 and FFA4 selective and PYY Y_1/Y_2-mediated. In A: concentration-response curve of varying concentrations of pinolenic acid (*n*=4-5). Points represent mean ± 1SEM. In B: basal changes in I_{sc} to TTX (100 nM, *n*=5), ANT825 (10 µM, *n*=5), AH-7614 (10 µM) + ANT825 (10 µM, *n*=5) and Y_1 and Y_2 antagonists (BIBO3304 + BIIE2046, *n*=5) compared with dH_2O (*n*=5), 0.1 % DMSO (*n*=5), 0.2 % DMSO (*n*=5) and 0.013 % DMSO (*n*=5), respectively. In C: pinolenic acid responses after VIP pretreatment in the absence and presence of TTX, ANT825, AH-7614+ANT825 or Y_1 + Y_2 antagonists. The responses of the internal control, exogenous PYY are shown in D. Bars represent the mean ± 1SEM. Statistical differences are shown as follows; $*P \leq 0.05$ and $***P \leq 0.001$ (Student's *t*-test).

3.16 Triple agonism of FFA1, FFA4 and GPR119, utilising the dual FFA1 and FFA4 agonist, pinolenic acid and selective GPR119 agonists

Having established co-agonism of FFA1, FFA4 or GPR119 in the descending colon mucosa was additive, the response to coincident triple receptor activation was next investigated, in an attempt to replicate the physiological activation of all three L cell receptors postprandially, *in vitro*. The responses to the dual FFA1 and FFA4 agonist, pinolenic acid, in combination with the full GPR119 agonist, PSN632408 or the selective GPR119 agonist, Cpd.16 were monitored for 20 min.

On its own, pinolenic acid slowly decreased I_{sc} and reached a maximal response within 8-10 min (Figure 3.16A). As expected, this duration was within the same time-frame reached by the maximal response to FFA1 (TAK-875) and FFA4 (Met-36) co-agonism, observed in the earlier study (seen previously in Figure 3.13A). The full GPR119 agonist, PSN632408, significantly decreased I_{sc} and its maximal response was observed within 8-10 min. Together, the combination of pinolenic acid and PSN632408 reduced the peak GPR119 PSN632408 response, however this was not significant when comparing the maxima. The combined response was reached within 6-8 min, 2 min faster than the individual agonist responses. Once the maximum of the combined response was reached, the I_{sc} rapidly returned to baseline. The time-course of the PSN632408 response was significantly different from the combined response at 12 and 15 min (Figure 3.16A).

The selective GPR119 agonist, Cpd.16 reached a maximal response within 5 mins, after which the I_{sc} rapidly returned to baseline I_{sc}. The time course of the response to the combination of Cpd.16 and pinolenic acid was not additive nor inhibitory, however showed similar kinetics to the time-course of the pinolenic acid response. Additionally, the combined response I_{sc} levels rapidly returned to baseline after it reached its maximum (Figure 3.16B). The absolute I_{sc} levels at the point of agonist addition were no different from one another in each cohort (Figure 3.16A-B insets). Exogenous PYY responses were no different in the presence of the various combinations of agonists (Figure 3.16C). However, the exogenous PYY response after PSN632408 appeared smaller (not significantly) in comparison to all other exogenous PYY responses (Figure 3.16C). This was attributed to a significantly lower absolute I_{sc} level before PYY application (after the PSN632408 response), in

comparison to the absolute I_{sc} levels after the Cpd.16 response and the Cpd.16 + pinolenic acid response (Figure 3.16C inset).

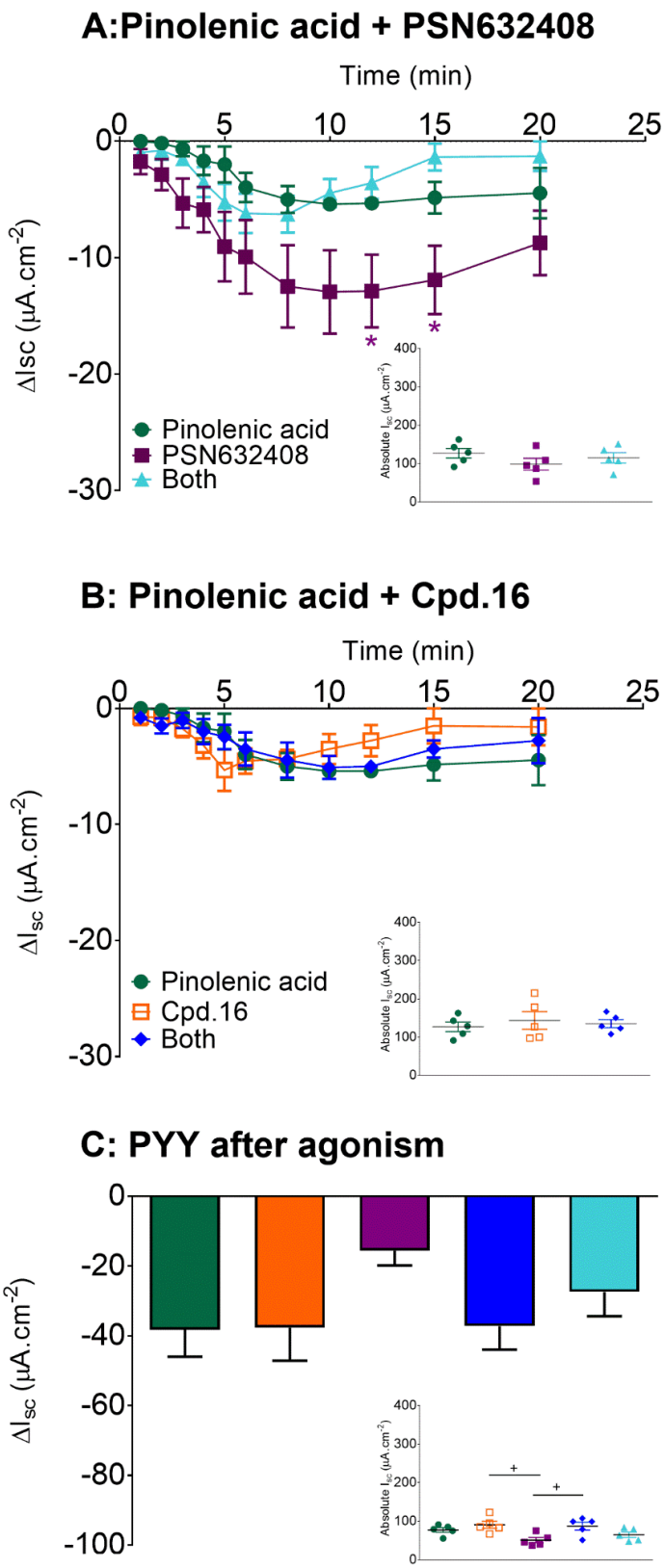


Figure 3.16: Triple agonism of FFA1, FFA4 and GPR119 after VIP pretreatment, utilising the dual FFA1 and FFA4 agonist, pinolenic acid and selective GPR119 agonists. In A: time courses of the anti-secretory responses induced by the dual

agonist, pinolenic acid (1 µM, *n*=5), the GPR119 full agonist, PSN632408 (10 µM, *n*=5) and the combination of both pinolenic acid and PSN632408 (Both, *n*=5). In B: time courses of the anti-secretory responses of pinolenic acid, Cpd.16 (1 µM, *n*=5) and the combination of pinolenic acid and Cpd.16 (Both, *n*=5). Notably, the pinolenic acid response time-course was duplicated in A and B to show the comparison between it, two independent GPR119 agonist responses and their combined responses with pinolenic acid, respectively. In C: PYY (10 nM, bl, *n*=5) control responses after agonist addition. Single points and bars represent the mean ± 1SEM. The insets in A - C display absolute I_{sc} values at the point of agonist addition. Symbols represent the number of observations and the black horizontal line displays the mean ± SEM. Statistical differences between each individual agonist response (at specific time-points) and the combined agonist response are shown as follows; * $P \leq 0.05$ (one-way ANOVA, with Bonferroni's *post hoc* test). The statistical differences between absolute I_{sc} cohorts before PYY addition (C) were analysed with a one-way ANOVA with a Bonferroni's post *hoc test* and shown as follows, $^{+}$ $P \leq 0.05$.

3.17 Caeco-colonic transit was inhibited by FFA1 and FFA4 agonists

Since FFA1 and FFA4 mucosal responses in the colon were mediated by PYY and this endogenous peptide was known to slow colonic transit (Savage et al., 1987; Lin et al., 1996; Maljaars et al., 2008; Tough et al., 2011), the ability of the FFA1 and FFA4 agonists were assessed to reduce colonic transit at single optimal concentrations (Figure 3.17A). In isolated colons, TUG424, TUG891, TAK-875 and Met-36 significantly decreased transit in comparison to vehicle-treated controls.

Since GW1100 inhibited FFA1 responses in colonic mucosa, the ability of GW1100 alone to increase basal faecal transit and reverse the effect of the FFA1 agonist on colonic transit was examined, *in vitro*. Figure 3.17B shows that GW1100 alone significantly increased basal colonic transit, again indicating endogenous FFA1 inhibitory tone. After 20 min pretreatment with the FFA1 antagonist, GW1100, the effect of TUG424 and TUG891 was assessed in the presence and absence of GW1100. In the presence of GW1100, the inhibitory effect on colonic transit of TUG424 was reversed, but GW1100 had no effect on TUG891 (FFA4) activity, showing FFA1 selectivity (Figure 3.17B). The positive control, loperamide HCl significantly

decreased faecal pellet propulsion in comparison to vehicle-treated controls (Figure 3.17C).

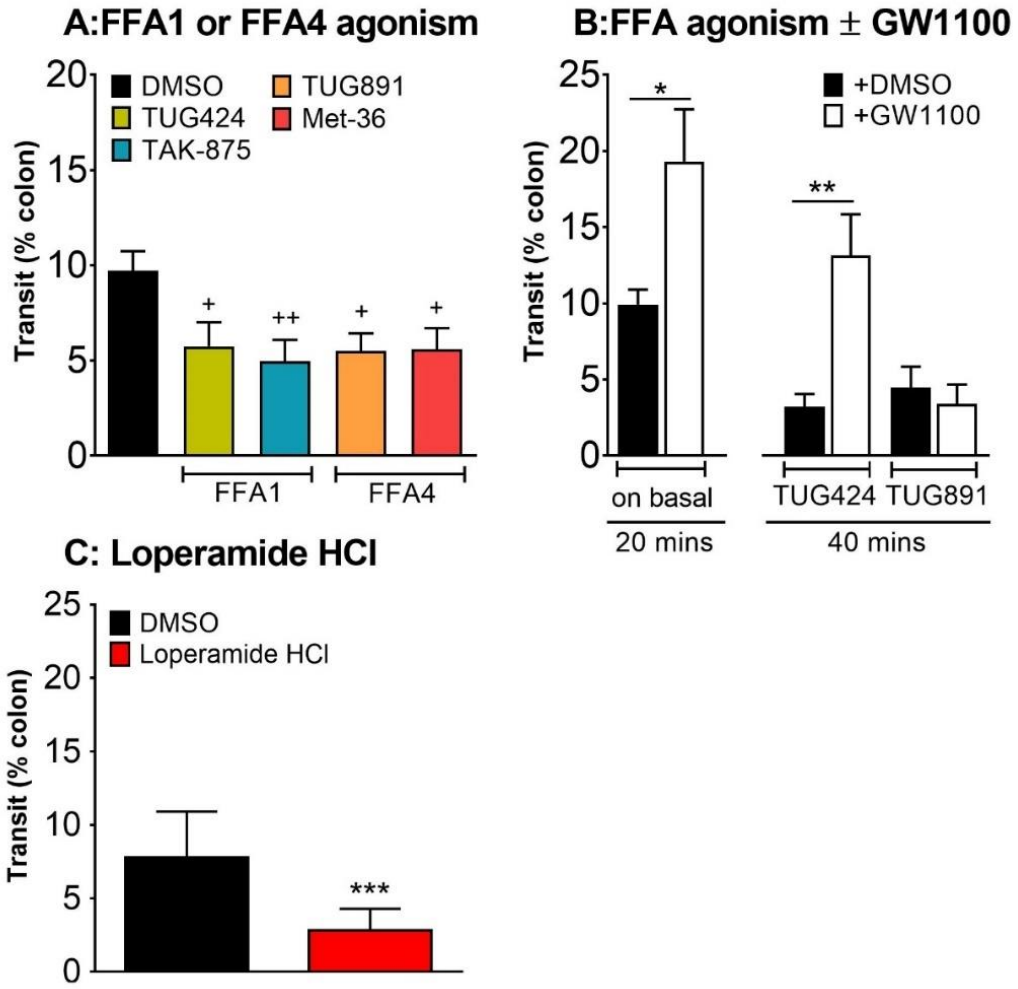


Figure 3.17: Colonic transit is slowed by FFA1 and FFA4 agonists in isolated colons *in vitro*. Colonic transit was slowed by TUG424 (300 nM, *n*=6), TAK-875 (300 nM, *n*=6), TUG891 (300 nM, *n*=6) and Met-36 (Met-36, 300 nM, *n*=6) in A at t =20 min. In B: the effects of FFA1 antagonist GW1100 (10 μM) and DMSO (0.1 %) are compared on basal faecal transit at t=20 min and on subsequent inhibition of transit following 20 min treatment with TUG424 (300 nM) or TUG891 (300 nM) at t=40 min (*n*=5, IR Tough data). In C: the positive control Loperamide HCl (10 μM, *n*=5) slowed colonic transit *in vitro* compared with DMSO (0.1 %, *n*=6). Each bar represents the mean + 1SEM. Statistically significant differences from controls were; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ (Student's *t* test) and $^{+}P \leq 0.05$ and $^{++}P \leq 0.01$ (one-way ANOVA with Dunnett's *post hoc* test).

3.18 Selecting a dose for TAK-875, ANT825 and Met-36 for in vivo experimentation

To establish a starting dose for each chosen compound (FFA1 agonist, TAK-875; the FFA1 antagonist, ANT825 and the FFA4 agonist, Met-36) selected to take forward into the *in vivo* GI motility studies, the following AZ in-house data provided insight (Figure 3.18A-F). Using this data, an optimum starting dose (and a higher dose if required) was chosen. The AZ in-house studies were performed in C57BL/6J mice (males and females), and therefore the results were comparable with the C57BL/6J mice used in this thesis. Mice received an intravenous (IV) or oral administration of each compound (various doses) and were monitored for 14-30 h. Blood samples were taken intermittently and analysed, measuring the total plasma concentration and the free plasma concentration of each compound. The administration (both routes) of each compound, TAK-875, Met-36 and ANT825, provided a maximal exposure (C_{max}) roughly around 1 h after administration. Therefore, in this thesis, the chosen compounds were administered (*po* or i.p.) 1 h prior to experimentation.

3.18.1 FFA1 agonist: TAK-875

The FFA1 agonist, TAK-875 produced by Takeda, has been used widely in the literature. In these studies, doses ranged from 3 mg/kg to 30 mg/kg (Tsujihata et al., 2011; Ito et al., 2013; Ito et al., 2016). In the *in vitro* studies in this thesis, TAK-875 provided an EC_{50} of 0.0676 µM (Figure 3.2A). Therefore, a dose was selected that provided a free plasma concentration around 0.1 µM of TAK-875 (around the EC_{80} concentration) (Figure 3.18A-B). Hence, with the support of AZ's drug metabolism and pharmacokinetics (DMPK) department, the starting *po*/i.p. dose for TAK-875 was 27 mg/kg.

3.18.2 FFA1 antagonist: ANT825

In this thesis, the FFA1 selective antagonist, ANT825 provided an IC_{50} value of 0.219 µM (seen previously in Figure 3.7C). Using AZ's in-house data, a dose of 29 mg/kg was selected for the initial *po*/i.p. dose of ANT825, as it provided a free-plasma concentration of 0.35 µM (an IC_{80} concentration of ANT825) (Figure 3.18C-D).

3.18.3 FFA4 agonist: Met-36

The FFA4 agonist, Met-36 provided an EC_{50} in this thesis of 0.015 µM (seen previously in Figure 3.2B). The *in vivo* motility studies required a free plasma concentration exposure of 0.024 µM of Met-36 (around the EC_{80} concentration). Hence, a starting *po*/i.p. dose of 6 mg/kg was selected however, this was later increased to 50 mg/kg (Figure 3.18E-F).

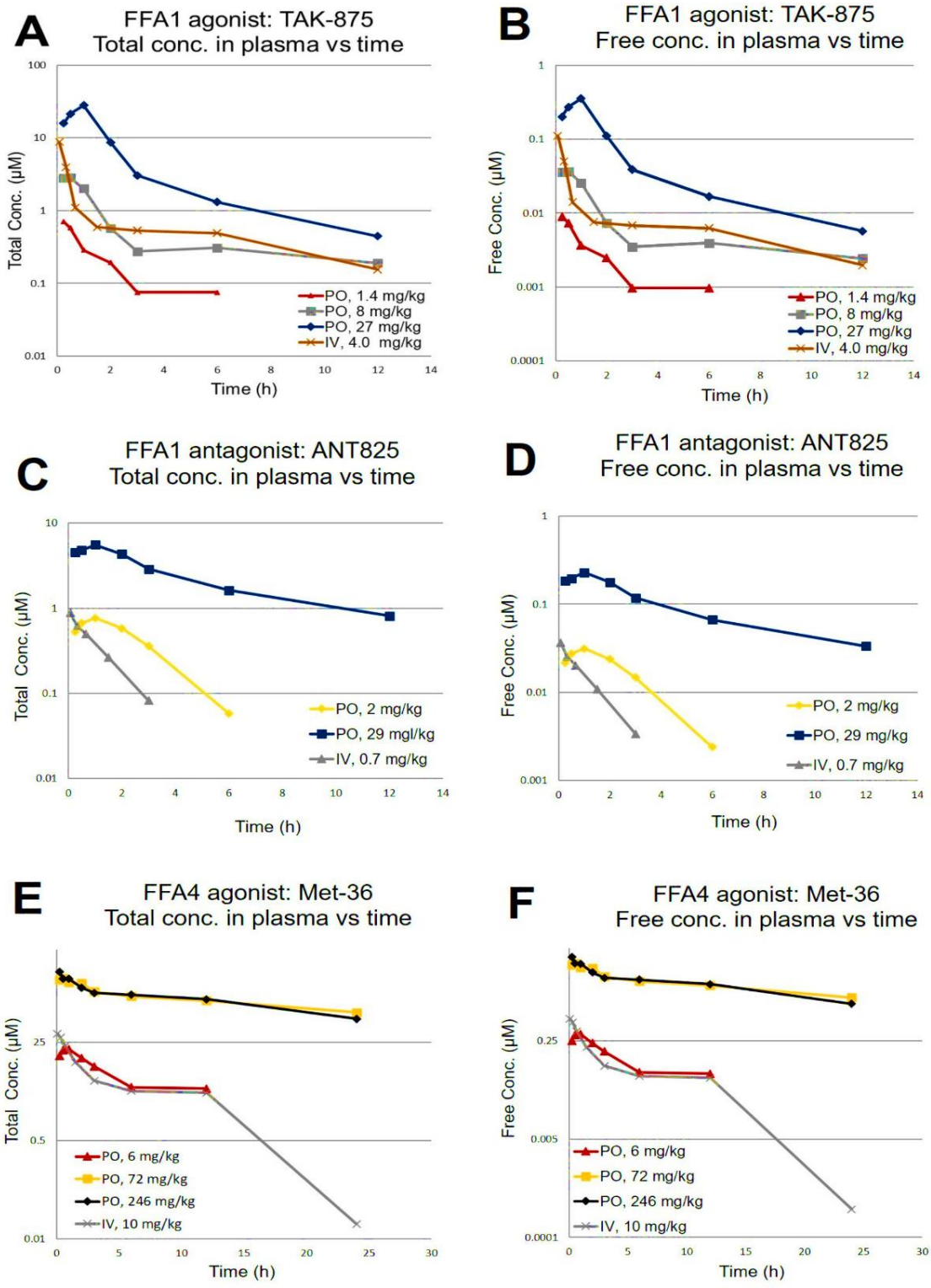


Figure 3.18: Time profiles of the total and free plasma concentrations of TAK-875, ANT825 and Met-36 in C57BL/6J mice. Total plasma concentration (µM) of the FFA1 agonist, TAK-875 (1.4 mg/kg, 8 mg/kg, 27 mg/kg (*po*) and 4 mg/kg intravenously (IV)) in A; the FFA1 antagonist, ANT825 (2 mg/kg, 29 mg/kg (*po*) and 0.7 mg/kg (IV)) in C and the FFA4 agonist, Met-36 (6 mg/kg, 72 mg/kg, 246 mg/kg

(*po*) and 10 mg/kg (IV)) in E, versus their free plasma concentrations (μM) in plasma (B, D, F respectively). Each point represents the time of blood sampling and the corresponding measurement of the total or free plasma concentration of agonist or antagonist.

3.19 Plasma glucose significantly decreased after a 16 h fast in mice

Before *in vivo* experimentation, mice were fasted for 16 h overnight. As FFA1, FFA4 and GPR119 agonist responses were glucose-sensitive *in vitro,* the blood glucose concentrations of a cohort of mice, were evaluated before and after a 16 h fast overnight. A blood sample was obtained from the tail vein, from each mouse, before and after fasting. The overall mean blood glucose concentration after fasting was significantly lower than the blood glucose concentration assessed before fasting (Figure 3.19). In summary, prior to *in vivo* experimentation, the C57BL/6J mice were hypoglycaemic after an overnight 16 h fast.

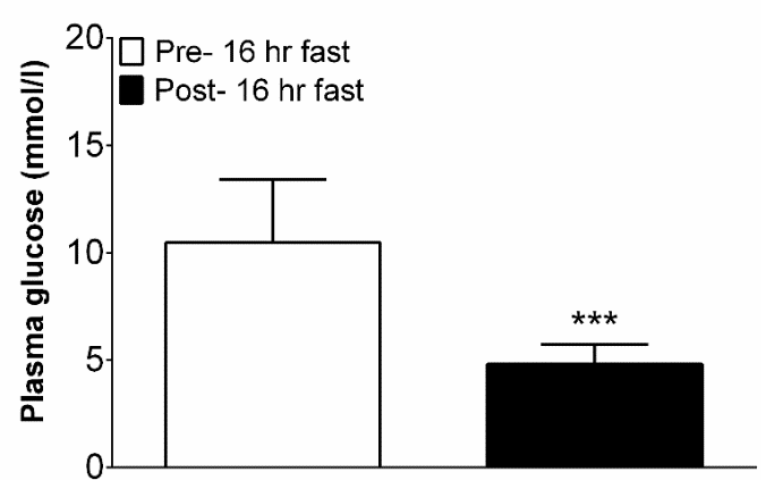


Figure 3.19: Plasma glucose concentrations pre- and post- 16 h fasting in C57BL/6J mice. Plasma glucose (mmol/l) concentrations before and after a 16 h overnight fast, prior to *in vivo* experimentation (*n*=7). The statistical difference between pre- and post-fasting is shown as ***$P \leq 0.001$ (Student's *t*-test). Bars represent mean + 1SEM.

3.20 TAK-875 and Met-36 responses were no different in 5.0 mM or 25.0 mM glucose in vitro

Having established that mice were hypoglycaemic after an overnight fast, the TAK-875 and Met-36 responses were assessed in descending colon mucosal preparations *in vitro*, bathed in KH containing 5.0 mM or 25.0 mM glucose on both sides of the reservoir (ap+bl). These glucose concentrations represented a hypoglycaemic (5.0 mM) and hyperglycaemic (25.0 mM) environment. Both TAK-875 and Met-36 responses were no different when the concentration of glucose in the KH was altered (Figure 3.20A). Phloridzin and PYY responses after agonist addition were also unaffected when the glucose concentration in the KH was altered (Figure 3.20B-C). In summary, although the mice used in this thesis were hypoglycaemic after fasting (before *in vivo* experimentation), FFA1 and FFA4 agonism was not affected when the glucose concentration in the KH was reduced (5.0 mM) to represent a hypoglycaemic environment *in vitro*. Additionally, FFA1 (TAK-875) or FFA4 (Met-36) responses in the presence of 5.0 mM or 25 mM glucose (in KH) were no different when compared with responses in an earlier experiment (Figure 3.11B & D, i.e. in 11.1 mM glucose).

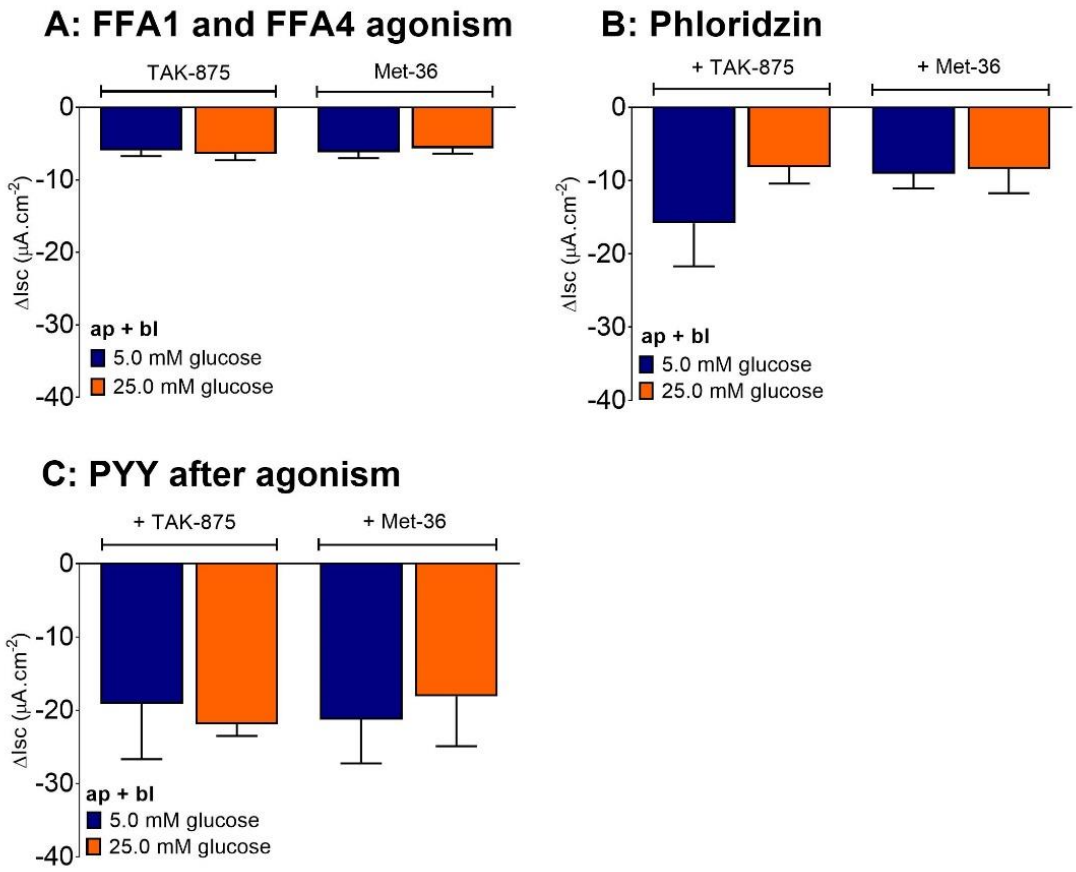


Figure 3.20: FFA1 and FFA4 responses were no different when the glucose concentration in the KH was altered to 5 mM or 25 mM. In A: Apical TAK-875 (200 nM, *n*=5) and Met-36 (100 nM, *n*=5) anti-secretory responses in descending colon mucosa, bathed in KH with a glucose concentration of 5.0 mM or 25.0 mM (ap + bl). Exogenous phloridzin (B) and PYY (C) responses (*n*=5) after agonist administration were unaffected in the presence of various concentrations of glucose. Bars represent mean - 1SEM.

3.21 Colonic bead propulsion was slowed by Met-36, not TAK-875 or ANT825 in vivo

As the FFA1 and FFA4 agonists (TAK-875 and Met-36, respectively) slowed transit in isolated colons *in vitro*, the ability of these agonists and loperamide HCl (positive control) to slow colonic transit was assessed *in vivo*, comparing *po* and i.p. administration. Whether ANT825 had the potential to reveal FFA1 tone was also investigated *in vivo*.

Oral administration of the positive control, loperamide HCl significantly slowed transit compared to its vehicle control. Two doses of Met-36 were evaluated orally; the lower dose had no effect on colonic transit, whereas the higher dose significantly slowed transit. Furthermore, oral gavage of TAK-875 and ANT825 had no effect on colonic transit (Figure 3.21A). Notably, the measured total plasma concentrations (as described in Methods & Materials) of TAK-875 (27 mg/kg, *po*) and Met-36 (50 mg/kg, *po*) were similar to that observed in AZ in-house *in vivo* studies (data not shown). Administration of loperamide HCl or Met-36 via i.p., significantly slowed colonic transit. Conversely, TAK-875 and ANT825 had no effect (Figure 3.21B). Notably, loperamide HCl (i.p.) slowed colonic transit significantly compared to all other compounds tested (Met-36, TAK-875, ANT825). As i.p. administration of loperamide HCl slowed transit more efficiently in comparison to oral gavage, this route of administration was used for each drug in the subsequent *in vivo* studies.

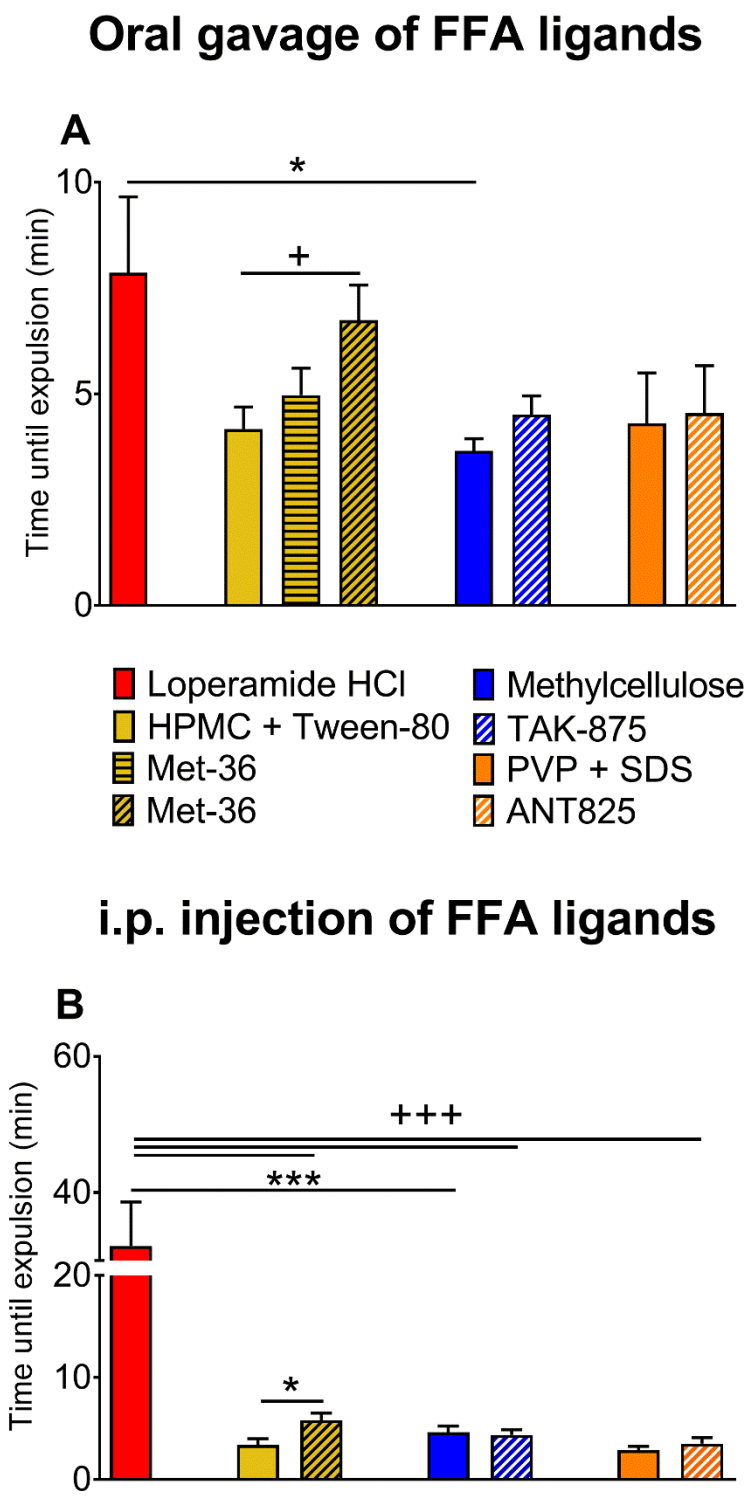


Figure 3.21: The effect of oral gavage versus i.p. administration of FFA ligands and loperamide HCl on colonic bead expulsion *in vivo*. The effect on colonic bead expulsion (expressed as time until bead expelled (min)) after oral gavage or i.p. administration of loperamide HCl (10 mg/kg, red bar, *n*=11) compared with its vehicle control, 0.5 % methylcellulose (blue bar, *n*=11); the FFA4 agonist, Met-36 at two doses: 6 mg/kg (yellow bar with black horizontal stripes, *n*=6) and 50 mg/kg (yellow

bar with black diagonal stripes, *n*=10) compared with their vehicle control, 0.5 % hydroxylpropyl methylcellulose (HPMC) + 0.1 % Tween-80 (yellow bar, *n*=10); the FFA1 agonist, TAK-875 (27 mg/kg, blue bar with white diagonal stripes, *n*=11) compared with its vehicle control (0.5 % methylcellulose, blue bar) and finally the FFA1 antagonist, ANT825 (29 mg/kg, orange bar with white diagonal stripes, *n*=7) compared with its vehicle (0.25 % polyvinylpyrrolidone (PVP) + 0.05 % sodium dodecyl sulphate (SDS), orange bar, *n*=7). Bars represent the mean + 1SEM. Statistically differences from controls were; * $P \leq 0.05$ and *** $P \leq 0.001$ (Student's *t*-test). Differences between the oral doses of Met-36 (*po*) from its control and additionally between loperamide HCl (i.p.) and the FFA drugs were; $^{+}P \leq 0.05$ and $^{+++}P \leq 0.001$ (one-way ANOVA with Dunnett's *post hoc* test).

3.22 Upper GI transit was increased by Met-36 in vivo

The effect (i.p.) of the chosen FFA agonists and loperamide HCl was investigated on UGIT, at the same doses utilised in the colonic motility *in vivo* study (section 3.21). Furthermore, as ANT825 revealed FFA1 tone *in vitro*, the effect of this antagonist was assessed *in vivo*. Mice were given a charcoal meal (as described in the Methods & Materials). The positive control, loperamide HCl significantly slowed UGIT as expected, compared to its vehicle control (Figure 3.22). Furthermore, loperamide HCl slowed UGIT significantly compared to all other compounds tested (Met-36, TAK-875, ANT825). Met-36 significantly increased upper intestinal transit, whereas TAK-875 and ANT825 had no effect (Figure 3.22).

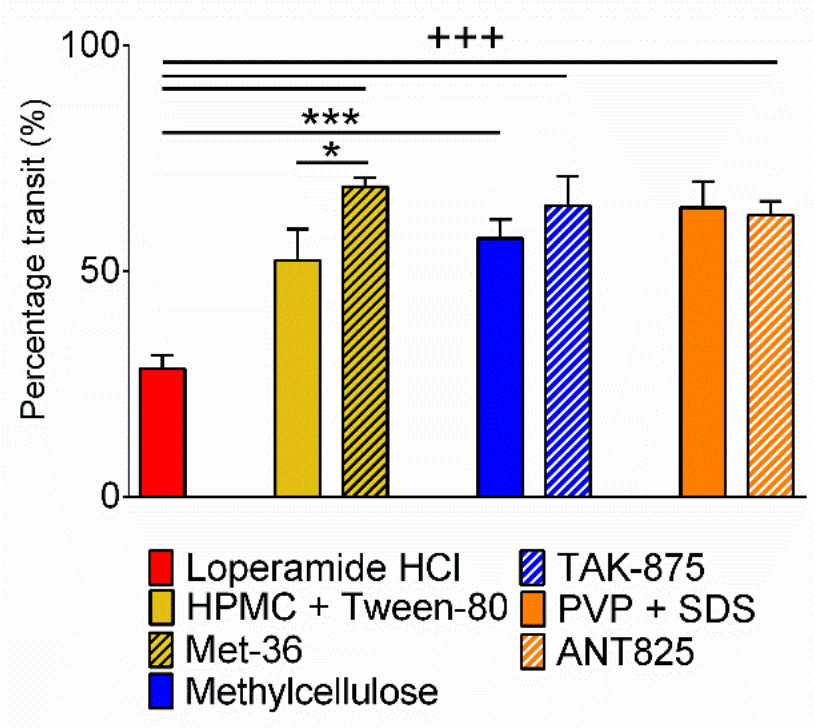


Figure 3.22: The effect of FFA ligands and loperamide HCl (i.p.) on UGIT *in vivo*. The intestinal transit of a charcoal meal (expressed as a percentage of the small intestine length) after the positive control, loperamide HCl (10 mg/kg, red bar, *n*=6) compared with its vehicle control, 0.5 % methylcellulose (blue bar, *n*=6); the FFA4 agonist, Met-36 (50 mg/kg, yellow bar with black diagonal stripes, *n*=6) compared with its vehicle control, 0.5 % HPMC + 0.1 % Tween-80 (yellow bar, *n*=6); the FFA1 agonist, TAK-875 (27 mg/kg, blue bar with white diagonal stripes, *n*=5) compared with its vehicle control (0.5 % methylcellulose, blue bar) and the FFA1 antagonist, ANT825 (29 mg/kg, orange bar with white diagonal stripes, *n*=5) compared with its vehicle (0.25 % PVP + 0.05 % SDS, orange bar, *n*=5). Bars represent the mean + 1SEM. Statistically differences from controls were; * $P \leq 0.05$ and *** $P \leq 0.001$ (Student's *t*-test). Differences between loperamide HCl and FFA drugs were; $^{+++}P \leq 0.001$ (one-way ANOVA with Dunnett's *post hoc* test).

CHAPTER 4
DISCUSSION

4.1 Bi-directional signalling of FFA1, FFA4 and GPR119 receptors

At the start of this PhD, the pharmacology of the FFA1, FFA4 and GPR119 receptors in native tissue was unclear, and very few functional studies had been performed interrogating their intestinal mechanisms. The first aim of this study was to determine the sidedness, potencies and efficacies of the novel selective agonists received from AZ (FFA1 agonists: JTT, TAK-875; FFA4 agonists: AZ423, Met-36 and GPR119 agonists: Cpd.16, Cpd.42) and compare them with commercially available agonists (FFA1 agonist: TUG424; FFA4 agonist: TUG891; FFA1 and FFA4 dual agonist: GW9508 and the GPR119 agonist: PSN632408). This study showed that FFA1, FFA4 and GPR119 agonism was independent of the surface of administration and the time-dependence of I_{sc} changes was similar upon luminal and serosal application. This suggested FFA1, FFA4 and GPR119 receptors were expressed on both the apical and basolateral epithelial surfaces, resulting in bi-directional signalling. Notably, all FFA1, FFA4 and GPR119 agonists (and antagonists) utilised in the present study were lipid-soluble, requiring DMSO as a vehicle. Thus, agonists added on the apical surface could activate receptors on the basolateral L cell domain and vice versa. However, no latency in I_{sc} was observed when comparing the apical versus basolateral time-courses of FFA1, FFA4 or GPR119 agonism. Thus, this increased the likelihood of FFA1, FFA4 and GPR119 receptor expression on both L cell surfaces, allowing bi-directional signalling to occur (Figure 4.1). Recently, Christensen *et al.* (2015) showed preferential but not exclusive vascular (basolateral) FFA1 evoked GLP-1 secretion, using endogenous and synthetic agonists in rat proximal small intestine. Luminal perfusion of the FFA1 agonist, AM-5262 (10 μM, 20 min) significantly increased portal vein total GLP-1 levels, at a concentration 10-fold higher than the concentration of AM-5262 (1 μM) perfused vascularly (Christensen et al., 2015). Despite the greater luminal concentration of AM-5262 (10 μM), it was still within the normal physiological luminal lipid range (mM). This revealed FFA1 receptors may also be expressed on the L cell apical domain in rat small intestine. A recent preliminary study has suggested exclusive vascular GPR119 agonism stimulated GLP-1 secretion in mouse and rat perfused small intestine, at a physiologically relevant concentration (1 μM) (Svendsen and Holst, 2016). However, no further findings have been published. Taken together, these studies suggested that the FFA1 and GPR119 receptors were preferentially expressed on the basolateral L cell domain in the small intestine. At odds with these studies, recently the novel first-in-class GPR119 water soluble

agonist, AR440006 (as well as OEA and 2-OG) elicited equi-effective apical and basolateral anti-secretory responses in mouse and human mucosa. Furthermore, liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis displayed only trace amounts of AR440006 (~2 nM) permeated the epithelial tight junctions, 10 mins after agonist addition (time of maximal agonism) (Tough et al., 2018b). This suggested GPR119 is expressed on apical and basolateral L cell domains in mouse and human colon, allowing bi-directional sensing, and this was consistent with the observed findings in this thesis. Differences between the findings in this thesis and the Christensen *et al.* (2015) study may be attributed to possible species differences in FFA1 and GPR119 signalling between rat intestine and mouse colon, respectively. Indeed, species variation exists between rat and mouse L cell MC_4 receptor enteroendocrine signalling (Panaro et al., 2014). Panaro *et al.* (2014) revealed the rat was not a suitable animal model to investigate L cell MC_4 receptor signalling as rat intestinal/colonic mucosa was unresponsive to the melanocortin, α-MSH (1 μM). α-MSH (10 nM or 1 μM) was also unable to elicit GLP-1 secretion in rat perfused small intestine. Conversely, α-MSH (1 μM) applied basolaterally in mouse descending colon mucosa (and human mucosa) caused an inhibition of epithelial ion transport and this functional effect was abolished in $MC_4^{-/-}$ mice (Panaro et al., 2014). Species variation also exists between rat and mouse (and human) Y_1/Y_2 receptor signalling. The complexity increases as rat Y_1/Y_2 receptor signalling also differs between GI regions, specifically the jejunum and colon (Cox and Cuthbert, 1990; Tough and Cox, 1996). In the rat jejunum, Y receptor signalling is preferentially Y_2-mediated, post-junctional and lacks Y_1-signalling. Conversely, in rat colonic mucosa there is a mixture of Y_1 and Y_2 signalling. Here, Y_1 receptors are neuronally expressed and Y_2 receptors are located post-junctionally (Tough and Cox, 1996). Similarly, in mouse and human colon there is a mixture of Y_1 and Y_2 signalling, however Y_1 receptors are predominantly found post-junctionally and expressed on epithelia whereas, Y_2 receptors are prejunctional (Cox et al., 2002; Hyland et al., 2003). Thus, Y-receptor signalling differences exist not only between the rat intestine and colon but also between rat and mouse colon. These key functional differences indicate Y_1/Y_2 receptor signalling in the mouse, not in the rat, more closely resemble human Y receptor signalling. Thus, caution should be taken when comparing rat and mouse signalling differences.

Luminal administered lipids or lipid metabolites i.e. FFAs have stimulated GLP-1 release in human (Carr et al., 2008; Little et al., 2005; Beglinger et al., 2010), mouse (Adachi et al., 2006) and rat (Dailey et al., 2014). Initial studies proposed FFA1 (Edfalk et al., 2008), FFA4 (Hirasawa et al., 2005) and GPR119 (Chu et al., 2008) were lipid-sensing receptors located on GLP-1 containing L cells, and that these receptors were responsible for sensing luminal lipid metabolites, to cause GLP-1 release. Indeed, over the years studies have shown that orally administered lipids or lipid metabolites can activate FFA1 (Edfalk et al., 2008; Xiong et al., 2013; Ekberg et al., 2016), FFA4 (Hirasawa et al., 2005; Ekberg et al., 2016) and GPR119 (Ekberg et al., 2016; Moss et al., 2016), to evoke GLP-1 secretion in WT mice *in vivo*. In rats, intraluminal application of the GPR119 endogenous agonist, OEA (10 µmol/l) but not intravenous administration (5 mg/kg), increased GLP-1 secretion *in vivo* (Lauffer et al., 2009). This OEA-mediated incretin effect demonstrated functional GPR119 agonism, and thus a possible indication of apical GPR119 receptors. The translatability of this luminal GPR119 response was observed in healthy human subjects as intrajejunal administration of the GPR119 endogenous agonist, 2-OG (Hansen et al., 2011) or oral administration of a lipid meal (C8-dietary oil, containing 2-OG) (Mandøe et al., 2015), also raised plasma total GLP-1 secretion. Taken together, apical administration was selected (Figure 4.1) to interrogate receptor function in further studies, as the concentration-dependent reductions in I_{sc} occurred within the same time frame as other agonists targeting apically located L cell GPCRs i.e. Cpd 1(FFA2) (Forbes et al., 2015), L-glutamine (calcium-sensing receptor) (Joshi et al., 2013), L-arginine (GPRC6A) (Alamshah et al., 2016) and the water-soluble agonist, AR440006 (GPR119) (Tough et al., 2018b).

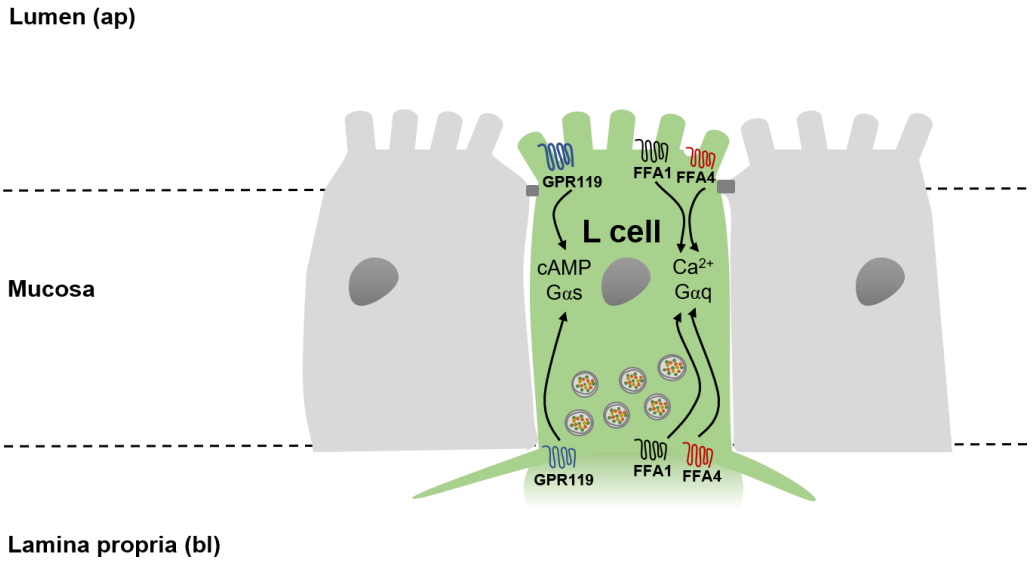


Figure 4.1: Proposed model of FFA1, FFA4 and GPR119 receptor sidedness in enteroendocrine L cells. FFA1, FFA4 and GPR119 receptors are present on both the apical (ap) and basolateral (bl) L cell epithelial domains, allowing bi-directional signalling in mouse descending colon mucosa.

4.2 The improved potency and selectivity of FFA1, FFA4 and GPR119 AZ agonists

4.2.1 Selective FFA1 agonists versus commercially available FFA1 agonists

The AZ FFA1 agonists, TAK-875 and JTT were equipotent and similarly efficacious in mouse native colonic mucosa. The potency of TAK-875 was similar to the EC_{50} of this compound (31 nM), in CHO cells expressing mouse FFA1 (AZ in-house data, Table 1.5). In another study, TAK-875 was more potent than FFA1 selective endogenous FFAs in a Ca^{2+} mobilisation assay, in CHO cells expressing mouse FFA1 (Yabuki et al., 2013). However, TAK-875 was not as efficacious as γ-linolenic acid, and therefore Yabuki *et al.* (2013) termed TAK-875 a partial FFA1 agonist. In this PhD thesis, TAK-875 (as well as JTT, TUG424, TUG891, AZ423 and Met-36) had a lower maximal response compared to the dual FFA1/FFA4 agonist, GW9508. However, due to the unavailability of known full selective FFA1 or FFA4 reference agonists, this PhD thesis had no evidence to suggest that these FFA1 agonists (TAK-875, JTT, TUG424) or FFA4 agonists (AZ423, Met-36 and TUG891) were partial agonists. More recently, TAK-875 exhibited a higher potency (EC_{50}=12.5 nM) than that observed in this PhD, in COS-7 cells expressing human FFA1 in an IP turnover

assay (Hauge et al., 2015), and this was similar to the potency of TAK-875 (EC_{50}=14 nM, fluorometric imaging plate reader (FLIPR)) at the human FFA1 receptor in the initial discovery study (Negoro et al., 2010). This modest difference in potencies between the findings in this thesis and those by Negoro *et al.* (2010) and Hauge *et al.* (2015), may be attributed to the transfection of the human, not mouse FFA1 receptor, of which its amino acid sequence differs by 17 % from the mouse FFA1 sequence (Table 1.3). In this PhD study, the EC_{50} of the second selective FFA1 agonist, JTT was more potent in native tissue than that obtained by AZ (Table 1.5). Furthermore, the EC_{50} of JTT was within the potency range of two lead compounds, compound 5 and 6 (between 1 nM -100 nM), published in a patent application by Japan Tobacco (NCT01699737). One of these compounds is thought to be JTT-851, and thus is consistent with the EC_{50} of JTT in this thesis.

The AZ FFA1 agonists were more potent but exhibited lower maximal responses than GW9508. GW9508 was reported to act as a dual agonist via FFA1 and FFA4, with 100x higher affinity for FFA1 than FFA4 (Briscoe et al. 2006). More recently, GW9508 has also been termed a partial agonist in comparison to two other FFA1 agonists (AM-1638 and AMG-6226) in A9 cells (a subline of mouse L cells), expressing FFA1 in an IP accumulation assay (Luo et al., 2012). Therefore, this dual FFA1 and FFA4 agonism with GW9508 may explain its slightly greater maximal response than the more selective FFA1 or FFA4 agonists used in this thesis. The EC_{50} of GW9508 obtained in this study was similar to the EC_{50} revealed in HEK293 cells, transfected with FFA1 or FFA4, in a Ca^{2+} mobilisation assay (Briscoe et al., 2006), and was not dissimilar from the potency observed in CHO cells expressing mouse FFA1 (Yabuki et al., 2013). The second commercially available FFA1 agonist, TUG424 showed similar kinetics as the selective FFA1 agonists, namely TAK-875 and JTT. In this present study, the EC_{50} of TUG424 was similarly potent to the EC_{50} of TUG424 (32 nM) observed in a label-free dynamic mass redistribution (DMR) assay. DMR is a measurement of the change in baseline refraction index upon receptor activation in a cell monolayer (Christiansen et al., 2008). Christiansen *et al.* (2008) first described TUG424 as a full FFA1 agonist but like GW9508 (Luo et al., 2012) and TAK-875 (Yabuki et al., 2013), TUG424 has also been termed a partial agonist in comparison to two other FFA1 agonists (AM-1638 and AMG-6226) in an IP accumulation assay in A9 cells expressing FFA1 (Luo et al., 2012).

FFA1 agonists are preferentially $G_{\alpha q/11}$-linked and recent evidence suggests that '$G_{\alpha q/11}$-only' FFA1 agonists (e.g. TAK-875) stimulate the release of incretin hormones, GLP-1 and GIP, with reduced efficacy compared to second generation FFA1 agonists. These second generation FFA1 agonists couple and signal via $G_{\alpha q/11}$ and $G_{\alpha s}$ pathways, to cause a more robust release of GLP-1 and GIP in murine colonic crypt cultures (Hauge et al., 2015; Hauge et al., 2017) and in mice *in vivo* (Hauge et al., 2015). The FFA1 agonists used in this present PhD study appear to be preferentially $G_{\alpha q/11}$-coupled as mucosal responses were transient in comparison with sustained $G_{\alpha s}$-coupled L cell signalling e.g. GPR119 (Cox et al., 2010; Patel et al., 2014) and MC_4 agonism (Panaro et al., 2014).

4.2.2 FFA4 selective agonists versus commercially available FFA4 agonists

In this PhD study, shown for the first time in native tissue, Met-36 and AZ423 were highly potent FFA4 agonists, more potent than the commercial FFA4 agonist, TUG891. Furthermore, all three FFA4 agonists were similarly efficacious. Recently, Met-36 (Sundström et al., 2017) and AZ423 (McCoull et al., 2017) exhibited EC_{50} values of 130 nM and 510 nM respectively, in CHO cells expressing mouse FFA4 in a DMR assay. In this PhD study, Met-36 and AZ423 appeared more potent in native tissue. In fact, the potency of AZ423 in HEK293 and CHO cells expressing mouse FFA4 (AZ-in house data, Table 1.5) was less potent than the potency observed in native tissue, demonstrating clear variation between cell-based assays and mouse native colonic mucosa. The commercially available FFA4 agonist, TUG891 exhibited an EC_{50} (62.5 nM (24.1 – 162.3)) within ranges previously published in a Ca^{2+} mobilisation assay at mouse FFA4 in HEK293 cells (Hudson et al., 2013). Notably, as only 3-fold TUG891 selectivity exists between mouse FFA4 and FFA1 receptors (Hudson et al., 2013), it is important to note that both FFA1 antagonists had no effect on the TUG891 response (300 nM) in this thesis.

In the past, FFA4 was shown to signal via $G_{\alpha q}$-coupled signalling pathways (Hirasawa et al., 2005). Recently, further complexity has been revealed as studies have suggested that FFA4 couples to $G_{\alpha i}$ signalling pathways in gastric ghrelin cells (Koyama et al., 2016), gastric somatostatin cells (Egerod et al., 2015) and pancreatic delta cells (Stone et al., 2014). In this thesis, the transient mucosal responses to the FFA4 agonists

resembled the same kinetics as the FFA1 agonists, and thus most likely signalled via $G_{\alpha q}$-linked pathways in mouse descending colon mucosa.

4.2.3 GPR119 selective agonists versus commercially available GPR119 agonists

The AZ GPR119 agonists, namely Cpd.16 and Cpd.42 were highly potent and exhibited low efficacy. The potencies of Cpd.16 and Cpd.42 were within ranges previously established in cAMP assays, in HEK293 cells expressing mouse GPR119 (Scott et al., 2012, Scott et al., 2014) (AZ in-house studies, Table 1.5). The commercially available full agonist, PSN632408 was highly efficacious and exhibited a low potency as previously shown (Overton et al., 2006; Cox et al., 2010). In comparison to the PSN632408 response, the selective GPR119 agonists appeared partial and displayed a maximal response similar to the GPR119 agonist developed by Prosidion, PSN-GPR119 (Patel et al., 2014). The maximum response to PSN-GPR119 was approximately 50 % lower than the maximum response to PSN632408 (Patel et al., 2014), as similarly observed with the AZ GPR119 agonists. Thus, like PSN-GPR119, the AZ agonists displayed partial GPR119 agonism. More recently, the GPR119 agonist, AR231453 also exhibited similar activity as PSN-GPR119, Cpd.16 and Cpd.42 (Tough et al., 2018b). This phenomenon of high potency but a low maximal response is indicative of a lower intrinsic ability of Cpd.16 and Cpd.42 to activate the GPR119 receptors once bound in comparison to PSN632408. Furthermore, the lower maximal responses of Cpd.16 and Cpd.42 may be attributed to enhanced receptor desensitisation/internalisation and will be discussed further in section 4.2.4 (below). In the present study, the sustained mucosal responses of PSN632408, Cpd.16 and Cpd.42 resembled $G_{\alpha s}$-signalling, as seen in previous L cell $G_{\alpha s}$-signalling investigations i.e. GPR119 (Cox et al., 2010; Patel et al., 2014; Tough et al., 2018b) and MC_4 (Panaro et al., 2014).

4.2.4 The selective and highly potent AZ FFA1, FFA4 and GPR119 agonists all displayed low maximal responses in mouse descending colon mucosa

The AZ FFA1 agonists (TAK-875, JTT) and FFA4 agonists (Met-36 and AZ423) exhibited lower maximal responses compared to the dual agonist, GW9508, whereas the AZ GPR119 agonists (Cpd.16 and Cpd.42) displayed partial activity in comparison to the PSN632408 response. Recently, Qian *et al.* (2014) demonstrated FFA1 agonist-

mediated internalisation was concentration and time dependent, with 300 nM - 1 µM linoleic acid causing approximately 40 % FFA1 internalisation, in HEK293 cells expressing FFA1. This agonist-mediated internalisation was β-arrestin 2 dependent (Qian et al., 2014). Further investigations by Mancini *et al.* (2015) in HEK293T cells (expressing GFP-tagged FFA1) revealed the FFA1 agonist, TAK-875 was a potent activator and full agonist in the recruitment of β-arrestin 1 (EC_{50} = 64.1 nM) and β-arrestin 2 (EC_{50} = 54.7 nM), compared to the endogenous FFA1 agonists, palmitate and oleate (Mancini et al., 2015). The activity of TAK-875 appeared partial in FFA1 $G_{\alpha q}$-coupled/Ca^{2+} signalling (Mancini et al., 2015) and Ca^{2+} flux assays (Yabuki et al., 2013) in comparison to other FFAs. Taken together, in these cell-based assays TAK-875 appeared to possess partial activity in $G_{\alpha q}$/Ca^{2+}-signalling and full agonism in β-arrestin 1 and 2 recruitment in comparison to palmitate and oleate (Mancini et al., 2015), clearly revealing signalling bias. In this PhD study, the low maximal response of TAK-875 and the other FFA1 agonist, JTT may be attributed to potential partial activity of these agonists in $G_{\alpha q}$/Ca^{2+} signalling.

Like the FFA1 receptor, FFA4 can also be internalised upon agonist stimulation (Hirasawa et al., 2005; Watson et al., 2012; Hudson et al., 2013). Hirasawa *et al.* (2005) demonstrated that 1 h incubation of the LCFA, α-linolenic acid (10 µM) caused internalisation of EGFP-tagged FFA4 in HEK293 cells. Hudson *et al.* (2013) went on further to investigate β-arrestin 2 recruitment and internalisation of GPR120S, the human short FFA4 isoform that closely resembles mouse FFA4. In this study, GW9508 activated GPR120S and this resulted in the recruitment of β-arrestin 2 (EC_{50} = 7.9 µM) in HEK293 cells. This potency was not dissimilar to the potency of GW9508 observed in their calcium mobilisation assay. Furthermore, GW9508 (100 µM) stimulated GPR120S internalisation with a half-life of 13 min and FFA4 endocytosis was predominantly non-recyclable. In the same study, the selective and potent FFA4 agonist, TUG891 (at 10 µM) induced robust FFA4 phosphorylation and internalisation of FFA4 in a HEK293 cell line expressing human FFA4, which was clearly observed within 10 mins (Hudson et al., 2013). The TUG891 EC_{50} (17.0 nM) obtained in the β-arrestin assay was similar to the EC_{50} of TUG891 in descending colon mucosa in this PhD study. In another study, the LCFA, DHA (EC_{50} = 1 µM) induced FFA4 phosphorylation in a HEK293 cell line expressing human FFA4 (short isoform). Furthermore, DHA (1 µM) stimulated human FFA4 internalisation within

15 min (Sánchez-Reyes et al., 2014). Recently, the FFA4 agonist, Met-36 efficiently recruited β-arrestin in U2OS cells expressing human FFA4, and obtained an EC_{50} value of 1400 ± 700 nM (Sundström et al., 2017). This is far less potent than the potency derived in this PhD study, which may suggest that 100 nM Met-36 may not recruit β-arrestin. The low potency of Met-36 observed by Sundström *et al.* (2017) may be attributed to the transfection of human, not mouse FFA4. Thus, this PhD study cannot rule out the possibility that Met-36 recruited β-arrestin to the FFA4 receptor, resulting in a low maximal response. Taken together, FFA4 agonism prompts receptor phosphorylation, β-arrestin recruitment and receptor internalisation. These mechanisms occur within a period of 10 -15 min and may underpin the low maximal response of the AZ selective FFA4 agonists utilised in this thesis.

Preliminary studies by Lauffer *et al.* (2009) demonstrated that when the human cell line, hNCI-H716 was incubated with PSN632408 (10 μM) for 6 h, this caused a significant decrease in subsequent OEA (70 %) or PSN632408 (50 %) induced GLP-1 secretion. This suggested GPR119 may undergo homologous desensitisation. For the first time, Hassing *et al.* (2016b) showed the GPR119 agonist, AR231453 was 166-fold more potent than the endogenous GPR119 agonist, OEA in a β-arrestin recruitment assay, utilising U2OS A_2 cells, expressing GPR119. Furthermore, recently AR231453 also displayed high potency with low maximal responses in mouse colon mucosa (Tough et al., 2018b). Thus, GPR119 partial agonists may recruit β-arrestin more potently than endogenous GPR119 agonists. In this thesis, GPR119 desensitisation was observed when the mucosa was pretreated with a high concentration of the GPR119 agonist, PSN632408, followed by a second application of PSN632408. However, a lack of FFA1 and FFA4 desensitisation was observed when the mucosa was pretreated with a high concentration of PSN632408, followed by the application of a FFA1 or FFA4 agonist. This indicated the GPR119 receptor may undergo homologous desensitisation but not cross-desensitisation, as shown previously by Lauffer *et al.* (2009). Notably, a limitation of this experimental design lies in the fact that there was no wash out period after the first PSN632408 addition. A lack of a response to the second addition of GPR119 agonist (PSN632408 or Cpd.42) may be attributed to first addition PSN632408 still occupying, activating and competing with the second addition of GPR119 agonist for occupancy of the GPR119 receptors. Therefore, a significant lower response would be observed to the second

GPR119 agonist as the receptors would already be occupied and activated. Recently, GPR119 homologous desensitisation was also observed with various potent synthetic GPR119 agonists and PSN632408 (at the same concentration used in this PhD study, 30 μM) in HEK293 cells (Zhang et al., 2014), which is consistent with the findings in this thesis.

Taken together, activation of FFA1, FFA4 and GPR119 with highly potent selective agonists, appears to recruit β-arrestin to each receptor. As β-arrestin proteins are essential in receptor desensitisation and internalisation (Luttrell and Lefkowitz, 2002), the low maximal responses displayed by the AZ agonists may be a result of bias signalling towards enhanced desensitisation and potential internalisation of these lipid receptors. This may be the reason tachyphylaxis was observed by the GPR119 clinical candidate, GSK1292263 (Kang, 2013). Repeated dosing of GSK1292263 in a Phase II trial was unable to reduce plasma fasting glucose or glucose $AUC_{(0\text{-}24h)}$ on day 13 or 14 in T2DM subjects, compared to placebo (Nunez et al., 2014). Therefore, this clinical trial was terminated due to lack of efficacy.

4.3 Regional variation of FFA1, FFA4 and GPR119 responses were more readily observed after VIP application

For the first time, the regional variation of FFA1, FFA4 and GPR119 agonism was assessed along the length of the GI tract on basal I_{sc} and after VIP pretreatment, in native intestinal mucosa utilising the AZ selective agonists. Agonism of FFA1, FFA4 or GPR119 revealed a similar response profile on basal I_{sc} and after VIP, however the changes in I_{sc} after VIP were greater and more readily observed. VIP allowed subsequent FFA1, FFA4 and GPR119 agonism to be more readily revealed, as the absolute I_{sc} after VIP was greater in comparison to the absolute basal I_{sc}.

The endogenous neuropeptide, VIP is a high affinity agonist of epithelial VPAC receptors (VPAC1 and VPAC2) (Laburthe et al., 2007). VPAC1 is highly expressed in the colon (proximal and distal) and the small intestine (Karacay et al., 2001; Jayawardena et al., 2017) whereas, VPAC2 is highly expressed in the colon (Harmar et al., 2004; Jayawardena et al., 2017). Radioligand binding studies have shown VIP binds to basolateral not luminal epithelial membranes in rat jejunum and rabbit ileum

(Dharmsathaphorn et al., 1983), and therefore VIP was applied basolaterally in this PhD study. As VPAC1 is the known predominant VIP receptor in the mouse intestine and colon, VIP responses in this thesis were presumably mediated via VPAC1 throughout the GI tract and VPAC2 may have had a role, specifically in the colon. The VPAC GPCRs are $G_{\alpha s}$-coupled, activation of which increases AC and accumulates cAMP, which elevates the I_{sc} (Vaudry et al., 2009). In this thesis, VIP pretreatment was used for enabling further characterisation of FFA1, FFA4 and GPR119 mucosal investigations.

4.3.1 FFA1 responses

In the present study, FFA1 mucosal responses after VIP appeared uniform along the length of the GI tract. This compared well with an initial study in mouse that utilised lacZ activity to show FFA1 expression was equally scattered along the GI tract, specifically in the duodenum, jejunum, ileum and colon (Edfalk et al., 2008). FFA1 responses after VIP in the mid-ileum were similar (modestly larger) to the responses observed in other regions of the GI tract. These findings correlated with mouse FFA1 mRNA relative expression findings, which indicated that FFA1 mRNA was greatest in the ileum and slightly lower in the distal regions of the GI tract (Symonds et al., 2015). Similarly, rat FFA1 mRNA (RT-PCR) was greatest in the ileum and equally expressed in duodenum, jejunum, caecum and colon (Itoh et al., 2003). More recently, FFA1 mRNA was also detected in human ileal mucosa (Tsukahara et al., 2015). Taken together, mouse and rat FFA1 mRNA appears to be highly expressed in the ileum, and this correlated with findings in this thesis, which showed FFA1 responses after VIP were greatest in this region too. As the expression and agonism of FFA1 appeared modestly larger in the ileum compared to FFA4 and GPR119 agonism, the FFA1 receptor may be the predominant lipid receptor in this area.

4.3.2 FFA4 responses

FFA4 mucosal responses were largest in the ascending colon and VIP pretreatment more readily revealed FFA4 agonism in the terminal ileum, which was modestly lower than agonism in the ascending colon. The regional variation of FFA4 agonism revealed by VIP was identical to the relative FFA4 mRNA expression profile along the length of the mouse GI tract (Symonds et al., 2015). Hirasawa *et al.* (2005) also revealed FFA4 mRNA expression (RT-PCR) was largest in the colon compared to the

ileum, in mouse and human (Hirasawa et al., 2005). Furthermore, in another study the relative expression of FFA4 increased along the length of the GI tract, with largest expression observed in the proximal mouse colon, which was consistent with findings in this PhD study (Ito et al., 2009). As FFA4 expression and FFA4 agonism was greatest in the proximal colon compared to FFA1 or GPR119 agonism, the FFA4 receptor may be the primary lipid receptor in this region.

4.3.3 GPR119 responses

Mucosal GPR119 anti-secretory responses increased in size along the length of the GI tract, on basal I_{sc} and after VIP, with the largest response observed in the descending colon, as displayed previously (Cox et al., 2010; Patel et al., 2014). This was also consistent with initial investigations demonstrating GPR119 expression was greater in the mouse colon than the small intestine, utilising a ^{32}P-labeled riboprobe containing an antisense mouse GPR119 sequence (Chu et al., 2008). Furthermore, the largest GPR119 response in the descending colon in this thesis correlated with the highest expression level of GPR119 mRNA (RT-PCR) in this region (Symonds et al., 2015). Recently, Moss *et al.* (2016) demonstrated that GLP-1 secretion to the synthetic GPR119 agonist, AR231453 and the endogenous GPR119 agonists, namely OEA and 2-OG from mouse primary cultures, was greatest in the colon, compared to the ileum and duodenum/jejunum (Moss et al., 2016). These findings were consistent with the GPR119 response profile observed in this thesis. Furthermore, Moss *et al.* (2016) revealed agonist-stimulated GLP-1 secretion was absent in colonic GPR119$^{-/-}$ cultures, indicating GLP-1 secretion was GPR119-mediated. Taken together, the findings in the present study, which were consistent with previous studies, confirm GPR119 agonism was greatest and more readily observed in the descending colon.

Lipids are predominantly absorbed and sensed in the duodenum (Iqbal and Hussain, 2009), a region where enteroendocrine L cells are relatively sparse (Sundler et al., 1993). In the present study, FFA1, FFA4 and GPR119 are predominantly expressed in the ileum, ascending colon and descending colon, respectively. Physiologically, in healthy individuals' dietary lipid metabolites are unlikely to be found in these distal intestinal regions (the ileum and colon). This does not mean these three receptors are not involved in lipid sensing in the upper intestine. In the proximal intestine, FFA1, FFA4 and GPR119 are expressed in CCK-containing I cells (Liou et al, 2011; Sykaras

et al., 2012) and GIP-containing K cells (Parker et al., 2009; Iwasaki et al., 2015). Thus, these three lipid receptors can be activated by dietary LCFAs in this region. Additionally, GPR119 is a well-known constitutively active receptor (Chu et al., 2007) and is also activated by endogenously synthesised OEA (Overton et al., 2006) and dietary-derived 2-OG (Hansen et al., 2011; Hassing et al., 2016a). Therefore, all three receptors in I or K cells could be activated as the luminal nutrient levels rise postprandially, and this would cause CCK and GIP secretion (see Table 1.2 for major functional effects of CCK and GIP).

As the greatest FFA1, FFA4 and GPR119 anti-secretory responses were observed in the distal small intestine and colonic regions, this suggests these lipid receptors may have a role in pathophysiological or drug/surgery-induced lipid malabsorption. In humans, colipase-deficiency (Hildebrand et al., 1982), pancreatic lipase-deficiency (Adrian et al., 1986), combined colipase/pancreatic lipase-deficiency (Ghishan et al., 1984), chronic destructive pancreatitis and coeliac disease (also known as tropical sprue) (Adrian et al., 1986) all result in lipid malabsorption and steatorrhea. Colipase is the pancreatic enzyme that allows pancreatic lipase to anchor onto the surface of triglycerides in the presence of bile, to initiate triglyceride hydrolysis (Erlanson-Albertsson, 1992; Lowe, 1997). Lipid absorption is reduced by approximately 50-75 % in colipase or pancreatic lipase deficiency (Hildebrand et al., 1982; Ghishan et al., 1984), allowing significant amounts of unabsorbed triglycerides and lipid metabolites to reach the distal intestine and colon. As FFA1, FFA4 and GPR119 agonism was greatest in these areas of the GI tract, one would expect the arrival of some LCFAs here (attributed to steatorrhea) to cause PYY release. Indeed, pre- and postprandial plasma PYY was increased in patients with steatorrhea induced by coeliac disease or chronic destructive pancreatitis. Furthermore, patients with steatorrhea exhibited increased weight loss and prolonged orocecal transit time (Adrian et al.,1986), which may have been attributed to the elevation in PYY.

Orlistat, a drug that inhibits the activity of pancreatic lipase by approximately 30 %, consequently causes steatorrhea and a 10-fold increase in faecal fat mass (Guerciolini et al., 2001). Long-term Orlistat usage has been shown to elevate pre-prandial PYY and GLP-1 levels and prolong orocecal transit time (Olszanecka-Glinianowicz et al., 2013). These effects may contribute to the modest weight loss these patients observe

and may suppress appetite and enhance insulin secretion with chronic drug use. Recently, steatorrhea was observed in some patients one-year post-RYGB surgery and this was associated with higher postprandial plasma PYY levels and prolongation of the ileal brake (O'Keefe et al., 2017). Thus, the presence of triglyceride metabolites in the distal intestine and colon presumably activates L cell FFA1, FFA4 and GPR119, which may lead to PYY release. Released PYY contributes to the ileal and colonic brakes (Spiller et al., 1984; Spiller et al., 1988; Lin et al., 1996) and is a defender against hypersecretion (Playford et al., 1990). Therefore, these three receptors are likely to be involved in the slowing of gut motility, which enhances lipid absorption and may prevent malnutrition in individuals experiencing steatorrhoea. Furthermore, functional agonism of these receptors may provide protection against diarrhoea and also contribute to the modest weight loss observed in Orlistat-treated individuals (Olszanecka-Glinianowicz et al., 2013).

The descending colon was selected as the optimal region for subsequent mucosal studies as this area of the GI tract is highly enriched with enteroendocrine PYY-containing L cells, which express all three lipid receptors. Moreover, this region also provided similar FFA1, FFA4 and GPR119 mucosal responses after VIP. As Y_1 receptors are highly enriched in the PYY-negative enterocytes surrounding the L cells (Mannon et al., 1999), this juxtaposition of PYY-containing L cells and enterocyte Y_1 receptors allowed PYY-Y_1 signalling to be interrogated. Therefore, the descending colon provided the greatest possibility of revealing specific L cell-mediated FFA1, FFA4 and GPR119 signalling mechanisms.

4.4 PYY-Y_1 but not PYY-Y_2 mediation of FFA1, FFA4 and GPR119 signalling in mouse colonic mucosa

For the first time, FFA1 and FFA4 agonist responses in the mouse colon were shown to be Y_1 receptor-mediated. The mucosal responses to the selective GPR119 agonist were also Y_1 receptor-mediated and not affected by Y_2 blockade, as shown previously utilising the following GPR119 agonists, namely PSN632408 (Cox et al., 2010) and PSN-GPR119 (Patel et al., 2014). This predominant PYY-Y_1 signalling mechanism suggested FFA1, FFA4 and GPR119 agonism did not involve enteric neurons. Pretreatment of the mucosa with TTX revealed neuronal tone attributed to the blockade of intrinsic neurogenic secretory activity. This was observed as a decrease

in I_{sc} as shown previously (Cuthbert and Hickman, 1985; Cox et al., 1988; Cox and Tough, 2002; Tough et al., 2011). Furthermore, FFA1, FFA4 and GPR119 responses were TTX-insensitive indicating FFA1, FFA4 and GPR119 activity was epithelial in origin and did not involve TTX-sensitive submucosal neurons. The TTX-insensitivity of GPR119 agonism has been revealed previously when investigating mucosal function, utilising the following GPR119 agonists, PSN632408 and PSN-GPR119 (Cox et al., 2010; Patel et al., 2014), and this was consistent with findings in this thesis. As a result, FFA1, FFA4 and GPR119 signalling in colonic mucosa was mediated by endogenous PYY acting on local epithelial Y_1 receptors ($G_{\alpha i}$-coupled). This inhibited AC activity and epithelial cAMP production, which limited anion secretion via CFTR (Figure 4.2) and caused an anti-secretory response. The epithelial Y_1 mediation and lack of any Y_2 (neuronal) involvement is consistent with the previous findings for other L cell-enriched receptors e.g. MC_4 (Panaro et al., 2014), FFA2 (Forbes et al., 2015) and the calcium-sensing receptor (Joshi et al., 2013).

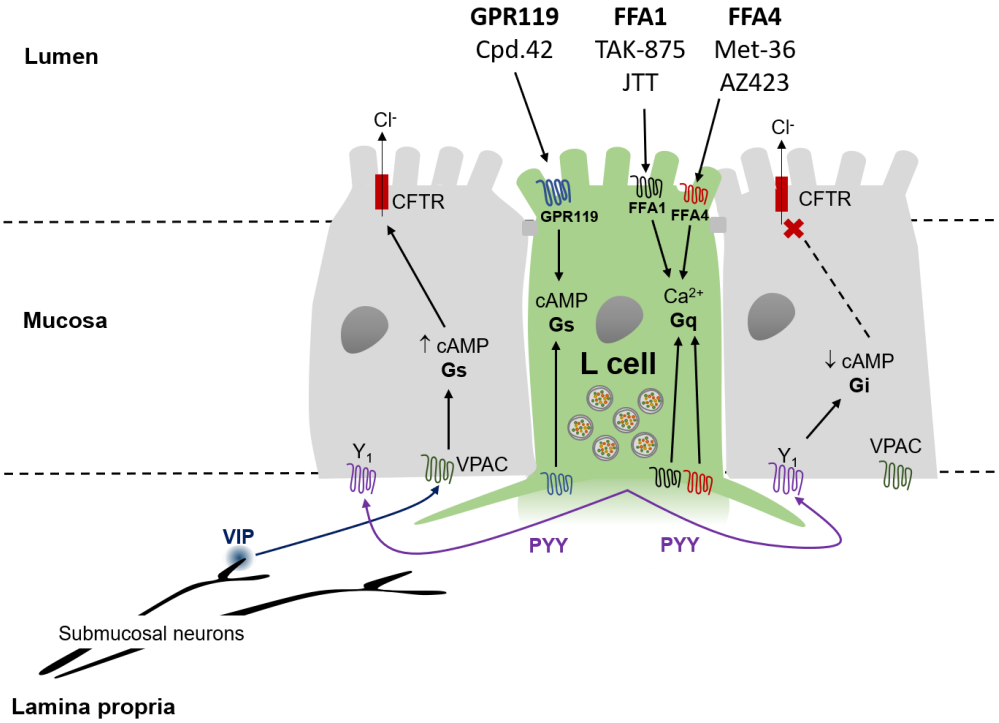


Figure 4.2: FFA1, FFA4 and GPR119 agonism is PYY Y_1-mediated resulting in inhibition of anion (Cl^-) secretion. Activation of apical (and basolateral, unlabelled for clarity) FFA1, FFA4 and GPR119 receptors with selective agonists (shown in figure) activates $G_{\alpha q}$ and $G_{\alpha s}$-signalling in the L cell to cause PYY release. PYY binds

epithelial Y_1 receptors ($G_{\alpha i}$) in a paracrine manner which consequently inhibits anion secretion via the CFTR channel. Thus, this lowers the I_{sc} and is observed as an anti-secretory response. VIP released from secretomotor submucosal neurons (or application of exogenous VIP) binds epithelial VPAC receptors (coupled to $G_{\alpha s}$), and this increases cAMP accumulation to cause anion (presumably Cl^-) secretion via CFTR.

4.4.1 Y_1 and Y_2 tonic activity

The Y_1 (BIBO3304) and Y_2 (BIIE2046) competitive antagonists revealed Y_1 and Y_2 tonic activity respectively, indicative of endogenous PYY-Y_1 and NPY-Y_2 activity. The degree of anti-secretory tone mediated by the Y_1 and Y_2 receptors was similar to that shown previously in mouse colon (Hyland et al., 2003; Hyland and Cox, 2005; Tough et al., 2011). Tough *et al.* (2011) demonstrated that Y_1 tone was significantly reduced (90 %) in $PYY^{-/-}$ and remained unaffected in $NPY^{-/-}$ colonic mucosal preparations. Conversely, the Y_2 tone was partially inhibited in both $PYY^{-/-}$ and $NPY^{-/-}$ preparations. The residual response observed in $PYY^{-/-}$ mucosae was TTX-sensitive. Moreover, Y_1 and Y_2 tone was abolished in double NPY/$PYY^{-/-}$ colonic mucosa. This confirmed that the Y_1 anti-secretory tone was epithelial in origin and PYY-mediated, whereas the Y_2 tone was neuronal and predominantly NPY-mediated (Hyland et al., 2003; Hyland and Cox, 2005; Tough et al., 2011). Thus, in this PhD, endogenous PYY and NPY activated Y_1 and Y_2 receptors to cause tonic Y_1 and Y_2 activity, respectively. Y_1 receptor tone may also be an indicator of tonic L cell PYY release. Indeed, Crivellato *et al.* (2002) demonstrated murine and human GI EECs undergo a slow secretory mechanism of stored peptides from the cell cytoplasm secretory vesicles, termed piecemeal degranulation (PMD). Utilising transmission electron-microscopy (TEM), this study clearly illustrated that the secretory granules lost density, structure and their matrices were loosely packed/diminished in mouse and human EECs. The peptides within these granules were completely or partially absent, leaving large translucent granules that did not fuse with one another or the plasma membrane (Crivellato et al., 2002; Crivellato et al., 2003). This revealed an active L cell mechanism, which consequently caused release of stored peptide hormones. Activation of L cell receptors by endogenous mediators or receptor constitutive activity may initiate PYY release and underpin the mechanism of PMD in these cells.

4.5 *Ex4 GLP-1 responses were largest in the ascending colon and a DPPIV inhibitor had the tendency to increase the stability of Ex4 in this region*

4.5.1 Ex4 GLP-1 responses were largest in the proximal colon

In this PhD study, the mucosal responses to the GLP-1 agonist, Ex4 were graded along the length of the colon (Tough et al., 2018a). The secretory response observed in the ascending colon declined in size towards the rectum and transitioned into an anti-secretory response in the descending colon. Furthermore, these mucosal responses were Ex(9-39)-sensitive, confirming the responses were GLP-1R-mediated, as shown previously (Joshi et al., 2013). In 2013, Joshi *et al.* demonstrated GLP-1 sensitivity was greater in the proximal colon compared with the descending colon. The proximal colon is the main region of fluid absorption (Barrett and Raybould, 2010). Notably, 4-20 % of patients on GLP-1 mimetics experience diarrhoea (Filippatos et al., 2014). Thus, greater GLP-1 signalling in the proximal colon may contribute to the diarrhoeal side effects experienced in these patients. This regional variation of GLP-1 responses raises the importance of future studies to stipulate the region of colon utilised.

4.5.2 The GLP-1R and CGRP mechanism

In the ascending colon, the Ex4 GLP-1 mucosal responses were TTX-sensitive (Tough et al., 2018a). Indeed, this confirmed that in the ascending colon, the GLP-1R was neuronally located. In the descending colon, Ex4 anti-secretory responses were too small to definitively confirm TTX-sensitivity. However, in both regions of the colon the Ex4 response was CGRP receptor-mediated. Approximately, 20 % of colonic submucosal cholinergic neurons co-stained for CGRP, and these ChAT/CGRP neurons were highly abundant in the ascending colon compared to the descending colon (Foong et al., 2014). Tough *et al.* (2018a) attributed this difference in the preponderance of ChAT/CGRP neurons in these colonic regions to the functional differences observed between the proximal and distal colon in their study. Thus, released GLP-1 activated GLP-1R on intrinsic submucosal neurons containing CGRP. The released CGRP activated epithelial CGRP receptors ($G_{\alpha s}$-coupled), which increased the production of cAMP and this opened anion channels, presumably CFTR and caused Cl^- secretion (Figure 4.3). Thus, in the ascending colon Ex4-induced a secretory response (Tough et al., 2018a). The finding that the GLP-1R was neuronally located in the colon was opposed by data published by Kedees *et al.* (2013). In their study, they showed GLP-1R localisation on mucosal epithelia in CD1 mouse ileum

and colon. However, the GLP-1R antibody utilised in their study was non-specific (Kedees et al., 2013). More recently, Richards *et al.* (2014) showed the localisation of the GLP-1R in different tissues utilising a transgenic glpr-cre mouse model that was crossed with fluorescent reporter strains. This allowed the expression of GLP-1R to be observed independent of a GLP-1R antibody. In their study, they showed GLP-1R expression in myenteric neurons and cell bodies in the small intestine and colon, which was consistent with findings revealed by Tough *et al.* (2018a). 19 % of colonic neurons contained NOS and a small percentage stained for CGRP and other markers (calretinin and calbindin) associated with IPANs (Richards et al., 2014). Thus, Tough *et al.* (2018a) proposed that these CGRP-containing sensory neurons were activated by Ex4 to cause CGRP release (Tough et al., 2018a).

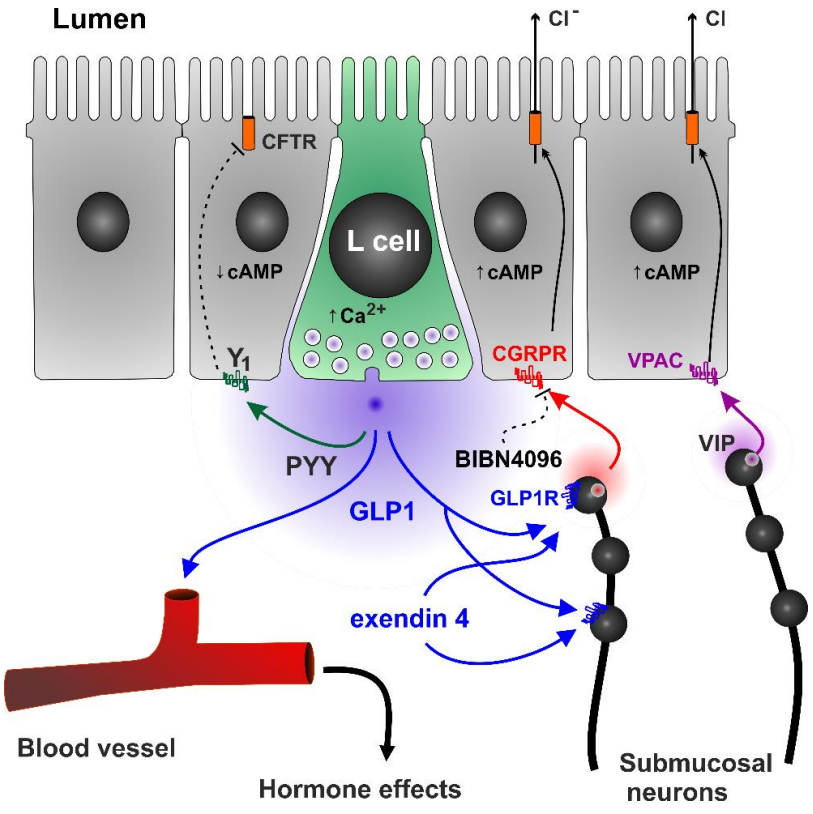


Figure 4.3: GLP-1/CGRP mechanism in the ascending colon. L cell released GLP-1 activates GLP-1R on intrinsic submucosal neurons to cause CGRP release. The released CGRP activates epithelial CGRP receptors ($G_{\alpha s}$-coupled) (blocked by CGRP antagonist, BIBN4096) which open CFTR to increase Cl^- secretion. Image from Tough *et al.* (2018a).

4.5.3 DPPIV inhibition had no effect on GPR119 agonism

While GLP-1 is the most common measured L cell hormone, in this PhD study no significant GLP-1 component was observed during FFA1, FFA4 or GPR119 agonism in descending colon mucosal preparations. Having established the largest GLP-1 sensitivity was found in the AC1-2 region of the ascending colon (Tough et al., 2018a), GPR119 agonism (utilising the full GPR119 agonist, PSN632408) was assessed in the presence of the DPPIV inhibitor, sitagliptin in this region and compared with mucosal responses in the descending colon. The DPPIV inhibitor prevents rapid degradation of the GLP-1 and PYY peptides (Mentlein et al., 1993a; Mentlein et al., 1993b), thus increasing their stability. In both regions of the colon in the presence of sitagliptin, the PSN632408 mucosal anti-secretory response was monophasic, not amplified by increased PYY stability and sitagliptin did not reveal any GLP-1 secretory component, as seen previously in mouse descending colon (Cox et al., 2010). Consistently, Cox *et al.* (2010) demonstrated that the conversion of PYY to $PYY_{(3-36)}$ was not significant in GPR119 agonism utilising PSN632408, and that the DPPIV inhibitor (Cpd.3) did not reveal a GLP-1 component to GPR119 agonism in mouse and human descending colon. More recently, the GPR119 agonist, AR231453 also displayed monophasic PYY Y_1-mediated responses and lacked a GLP-1 response (Tough et al., 2018b). Notably, in human mucosa blockade of Y_1 and Y_2 receptors revealed a GLP-1 component to the PSN632408 response (Cox et al., 2010). This suggested GLP-1 and PYY are co-released from enteroendocrine L cells and that the GLP-1 component was too small to be revealed in the presence of PYY Y_1 and Y_2 agonism in human tissue. Thus, the lack of a GLP-1 secretory response to GPR119 agonism (and FFA1 or FFA4 agonism) in this thesis, was most likely attributed to the low levels of the GLP-1R expressed in submucosal neuronal fibres in the mouse colon (Richards et al., 2014), in comparison to the high expression levels of the epithelial Y_1 receptors in this region (Mannon et al., 1999). This contrasting ratio of receptor expression could limit GLP-1 signalling in the colon, as PYY-Y_1 epithelial responses would be more readily observed. The DPPIV inhibitor, sitagliptin had the tendency to increase the stability of the Ex4 mucosal response in the ascending colon, indicated by the apparent increase in I_{sc} compared to the control. The small Ex4 mucosal response observed in the descending colon and the lack of effect of sitagliptin here, was most likely attributed to fewer GLP-1R containing ChAT/CGRP neurons in this region, in comparison to the ascending colon (Foong et al., 2014; Richards et al., 2014).

The lack of a GLP-1 secretory component associated with GPR119 agonism was at odds with previous studies in cell-based assays (*ex vivo)* and *in vivo* studies, which have shown GPR119 agonism robustly stimulates GLP-1 release (Chu et al., 2008; Mace et al., 2012; Patel et al., 2014; Hauge et al., 2015; Ekberg et al., 2016; Hassing et al., 2016a; Moss et al., 2016; Hauge et al., 2017). Furthermore, PSN-GPR119 (Patel et al., 2014) and OEA (Mace et al., 2012) have been shown to increase GLP-1, GIP and PYY secretion in the presence of 5 mM glucose, in rat isolated intestinal loops. In this thesis, the AZ selective GPR119 agonists, Cpd. 42 and Cpd. 16 did not exhibit a GLP-1 secretory component to their responses *in vitro*. However, in previously published studies both Cpd.42 and Cpd.16 increased plasma total GLP-1 levels in mice *in vivo,* an effect that was elevated in the presence of sitagliptin (Scott et al., 2012; Scott et al., 2014). These GPR119 preclinical studies have translated in human subjects as administration of GPR119 activators, 2-OG or C8-dietary oil (contains 2-OG) have caused incretin secretion *in vivo* (Hansen et al., 2011; Mandøe et al., 2015). Thus, GPR119 agonism caused robust GLP-1 secretion in rodents and humans, but this was not observed in colonic mucosa in this PhD study *in vitro*.

The absence of a GLP-1 secretory response may also be attributed to the recent discovery of differential peptide release from enteroendocrine L cells (Habib et al., 2012; Cho et al., 2014; Cho et al., 2015; Grunddal et al., 2016). In the past, various EECs were named according to their hormonal content using a letter code (L, K, I, N etc.). Using this system, L cells contained GLP-1, GLP-2, PYY and OXM, K cells expressed GIP, I cells contained CCK and N cells secreted NTS. Over the years, there has been a rise in evidence to suggest this system is less uniform than previously thought. In 2012, Habib *et al.* (2012) utilised immunohistochemical analysis and FACs analysis to identify subsets of EECs. Here, colonic L cells contained mRNA for PYY and GLP-1, whereas L cells in the small intestine contain mRNA for NTS, CCK, GIP and GLP-1 (Habib et al., 2012). In 2015, Cho *et al.* investigated peptide localisation in colonic L cells in the mouse. In addition to the classic GLP-1 and PYY containing L cells, various other peptide combinations were observed using triple labelling immunochemistry: GIP only cells, GIP/GLP-1, GLP-1 only, PYY only cells (Cho et al., 2015). Cho *et al.* (2014) demonstrated that GLP-1 and PYY were also discovered in separate storage organelles in the same EEC, implying differential release may occur. More recently, Grunddal *et al.* (2016) showed separate storage of

PYY, GLP-1, and NTS in murine distal small intestine, using super resolution structural illumination microscopy. This differential PYY and GLP-1 release between L cells and peptide packaging within the same L cell vesicles, may contribute to the reason GPR119 agonism appeared to be mediated by a PYY Y_1 epithelial mechanism, without the involvement of GLP-1, as shown previously with FFA2 agonism (Forbes et al., 2015). Forbes *et al*. (2015) revealed FFA2 agonism in descending colon mucosa was PYY-Y_1, not GLP-1-mediated *in vitro*. This predominant PYY-Y_1 signalling mechanism and the absence of a GLP-1 response to FFA2 agonism *in vitro* was also consistent with FFA1 and FFA4 signalling in this PhD study *in vitro*.

4.5.4 FFA1 and FFA4 responses appeared GLP-1 independent

Originally, it was reported that FFA1 and FFA4 were highly expressed in GLP-1-containing L cells (Hirasawa et al., 2005; Edfalk et al., 2008). Edfalk *et al.* (2008) demonstrated a HFD caused a rise in plasma GLP-1 (and GIP) concentrations in mice and these peptide secretions were reduced to basal levels in $FFA1^{-/-}$ mice. This indicated a definitive role for the FFA1 receptor in GLP-1 secretion. Later, Xiong *et al.* (2013) revealed a gavage of corn oil (containing linolenic and linoleic acid) evoked GLP-1 secretion and this was abolished in $FFA1^{-/-}$ mice, further confirming a major role for FFA1 in GLP-1 release (Xiong et al., 2013). Disappointingly, while the FFA1 clinical candidate, TAK-875 improved glucose tolerance in diabetic Japanese human subjects, it did not evoke any incretin secretion (GLP-1 and GIP) *in vivo* (Kaku et al., 2015). Recently, mouse primary culture *ex vivo* preparations have been utilised to assess GLP-1 release upon application of endogenous LCFA or synthetic agonists. In mouse colonic primary cultures, TAK-875 (1 µM) modestly elevated total GLP-1, 1.5-fold above basal secretion, an effect that was eliminated in $FFA1^{-/-}$ cultures. This modest increase in GLP-1 secretion was also observed in WT mice and was absent in $FFA1^{-/-}$ mice *in vivo* (Hauge et al., 2015). In this thesis, the responses to the selective FFA1 agonists, TAK-875 and JTT (including the commercially available TUG424) were monophasic and lacked a GLP-1 component in descending colon mucosa. Thus, FFA1 responses in this region of intestine appeared to be independent of a GLP-1-mediated mechanism, which was consistent with the lack of TAK-875-induced incretin secretion observed in clinical trials. Indeed, Ekberg *et al.* (2016) recently showed that the FFA1 receptor has a relatively small role in olive oil evoked incretin secretion (GLP-1 and GIP), by demonstrating loss of function in $FFA1^{-/-}$ mice.

Furthermore, TAK-875 was not an efficacious secretagogue of GLP-1 in *ex vivo* primary culture colonic preparations (Ekberg et al., 2016). This suggested FFA1 on its own, did not have a major role in incretin secretion. These findings and the lack of FFA1 GLP-1 response observed in this PhD study may also be attributed to potential partial $G_{\alpha q}$-signalling activity of TAK-875 (Yabuki et al., 2013; Mancini et al., 2015) and TUG424 (Luo et al., 2012). Unfortunately, the potential partial activity of these compounds could not be tested due to the unavailability of known full FFA1 reference agonists. Xiong *et al.* (2013) demonstrated a FFA1 partial agonist, AM-6331 did not stimulate GLP-1 release *ex vivo* or *in vivo* in comparison to a full FFA1 agonist, AM-8182 (Xiong et al., 2013). Thus, in this thesis, a lack of a GLP-1 component to FFA1 agonism may have been attributed to the partial activity of these AZ FFA1 agonists. Whether the latter applies to FFA4 and GPR119 partial and full agonists has yet to be elucidated.

The FFA4 receptor was identified in GLP-1 containing cells as the second characterised LCFA receptor (Hirasawa et al., 2005). Hirasawa *et al.* (2005) measured portal vein and inferior vena cava plasma GLP-1 in response to α-linolenic acid in mice *in vivo*, implicating FFA4 in GLP-1 release. However, α-linolenic acid also acts via FFA1 (Hara et al., 2011) and this experiment was not carried out in $FFA4^{-/-}$ mice to demonstrate GLP-1 release was FFA4, not FFA1-mediated (Hirasawa et al., 2005). Indeed, there has been an increasing body of evidence to suggest FFA4 has a relatively minor role, if any, in GLP-1 release in rodents (Xiong et al., 2013; Paulsen et al., 2014; Ekberg et al., 2016). In the present study, like FFA1 agonism, the mucosal responses observed with the FFA4 selective agonists, namely Met-36, AZ423, were monophasic without any indication of a GLP-1 component. These findings were consistent with those observed by Xiong *et al.* (2013) displaying corn oil-induced GLP-1 responses were not eliminated in $FFA4^{-/-}$ mice *in vivo*, indicating no role for FFA4 in GLP-1 secretion (Xiong et al., 2013). Additionally, Ekberg *et al.* (2016) recently demonstrated a previously efficacious FFA4 agonist, Metabolex-204, had no effect on GLP-1 release in *ex vivo* mouse primary colonic cultures and has a minor role (not significant) in GLP-1 release *in vivo* (Ekberg et al., 2016). Taken together, the findings in this PhD study were similar to those previously published and suggested physiologically FFA4 agonism has a minor role if any, in GLP-1 secretion.

4.6 FFA1 and FFA4 antagonists revealed AZ agonists were highly selective

4.6.1 ANT825 and GW1100 were selective FFA1 antagonists

The potency of the AZ FFA1 competitive antagonist, ANT825 was not dissimilar from its potency (EC_{50} = 158.5 nM) obtained in a HEK293 cell line expressing human FFA1, measuring inositol monophosphate (Waring et al., 2015). In this thesis, the commercially available FFA1 antagonist, GW1100 (IC_{50} = 1.0 μM; (Briscoe et al., 2006)) abolished TUG424 responses but not TUG891 responses, showing FFA1 selectivity, as seen previously (Briscoe et al., 2006). Like GW1100, ANT825 abolished FFA1 responses but both FFA1 antagonists also partially inhibited GW9508 responses, indicating that at this concentration (1 μM); GW9508 exerted dual agonism via FFA1 and another receptor. ANT825 had no effect on FFA4 or GPR119 agonism confirming the FFA1 selectivity of this FFA1 antagonist.

4.6.2 *AH-7614 was a selective FFA4 antagonist in mouse descending colon*

Interrogation of the selectivity of the FFA4 agonist, Met-36, with the FFA4 antagonist, AH-7614 in native mouse descending colon, revealed a concentration-dependent inhibition of the Met-36 response. Notably, Met-36 is known to possess 100-fold higher selectivity for FFA4 in comparison to FFA1 (Stone et al., 2014). This inhibition of FFA4 agonism was not as potent as the potency of AH-7614 observed in the original study (IC_{50} = 7.9 nM), utilising U2OS human osteosarcoma cells expressing mouse FFA4 (Sparks et al., 2014), and was not dissimilar from the IC_{50} of AH-7614 revealed at the mouse FFA4 receptor in the presence of TUG891 (500 nM), in a β-arrestin recruitment assay in HEK293T cells (IC_{50} = 11.7 nM (10.2 – 13.5)) (Watterson et al., 2017). These differences may be attributed to the fact that antagonist IC_{50} measurements in different assays/studies were not directly comparable as the concentration of agonist utilised often varied between studies. In this PhD study, 10 μM AH-7614 did not completely inhibit FFA4 agonism (Met-36). This finding was consistent with Watterson *et al.* (2017) demonstrating that FFA4 agonism (TUG891, 3 μM) induced FFA4 internalisation, and this effect was not completely inhibited in the presence of 10 μM AH-7614. This indicated that AH-7614 displayed negative allosteric modulator (NAM) properties when inhibiting the functional effect of TUG891 (Watterson et al., 2017). Thus, Watterson *et al.* (2017) termed AH-7614 a NAM. Having established the GW9508 response was partially FFA1-mediated, the GW9508 response was investigated in the presence of the FFA4 NAM, AH-7614 in

combination with and the FFA1 antagonist, ANT825. The GW9508 response was significantly reduced, more so than the GW9508 response in the presence of ANT825 alone. Thus, GW9508 exerted dual FFA1 and FFA4 agonism, in the mouse descending colon. Originally Briscoe *et al.* (2006) showed GW9508 raised $[Ca^{2+}]_i$ in HEK293 cells expressing the human FFA4 receptor, a functional effect that was not affected by the FFA1 antagonist, GW1100 (Briscoe et al., 2006), demonstrating GW9508 was also FFA4 selective. In this PhD study, GW9508 exerted dual agonism via FFA1 and FFA4 in mouse descending colon mucosa.

4.6.3 FFA1 antagonism revealed FFA1 mucosal tone

The FFA1 antagonists, namely ANT825 and GW1100, revealed for the first time a degree of endogenous FFA1 tonic activity in colonic mucosa. Furthermore, ANT825 revealed FFA1 tone was uniform along the length of the GI tract, as similarly observed in the tissue survey of FFA1 agonism in this thesis (Results, Figure 3.3). The degree of FFA1 tonic activity was similar to that observed with the MC_4 antagonist, HS014 (Panaro et al. 2014) and the competitive GPR119 antagonist, AR436352 (Tough et al., 2018b). Since endogenous FFA1, MC_4 and GPR119 agonism stimulates PYY release from L cells, blockade of these receptors inhibits endogenous PYY release and Y_1 activation, causing a consequent increase in I_{sc}. Thus, FFA1 agonism (as well as GPR119 or MC_4 agonism) by endogenous mediators may underpin the tonic activity observed in the mucosa. Tonic activity may also be an indicator of a constitutively active receptor. Stoddart *et al.* (2007) reported FFA1 appeared as an apparent highly constitutively active receptor in HEK293 cells, utilising a 5'-O-(3-[^{35}S]thio)triphosphate([^{35}S]GTPγS) binding assay. However, bovine-serum albumin (BSA) reduced the basal [^{35}S]GTPγS binding in the immunoprecipitates and this was attributed to the binding of BSA to FFAs. Furthermore, binding was also eliminated by the FFA1 antagonist, GW1100 and the $G_{\alpha q}/G_{\alpha 11}$ inhibitor, YM254890 (Stoddart et al., 2007). As a result, the 'FFA1 constitutive activity' was more likely attributed to the binding of an unidentified endogenous mediator. MCFA/LCFA are the endogenous activators of FFA1 (Itoh et al. 2003). Thus, lipid metabolites hydrolysed during food intake (chow) that are present in the lamina propria, or an unidentified endogenous ligand may be responsible for the observed FFA1 tonic activity.

4.6.4 The absence of FFA4 tone may be attributed to FFA4 agonism in L cells and enterocytes

Unlike, the FFA1 antagonists (ANT825 and GW1100), this PhD study revealed for the first time that the FFA4 NAM, AH-7614 had no effect on basal FFA4 tone. The lack of FFA4 tone was surprising as most endogenous LCFAs non-selectively activate both FFA1 and FFA4 (Briscoe et al., 2003; Briscoe et al., 2006; Hara et al., 2011). While FFA4 was originally identified in GLP-1 containing L cells (Hirasawa et al., 2005), two independent groups have revealed FFA4 is highly expressed in mouse and rat enterocytes, as well as L cells (Figure 4.4) (Paulsen et al., 2014; Ekberg et al., 2016). Assuming FFA4 was broadly expressed in the mucosal preparations in this PhD study and an unidentified endogenous ligand non-selectively activated both FFA1 and FFA4, the FFA4 receptor may have been tonically active in L cells (like the FFA1 receptor) and the surrounding enterocytes. Theoretically, tonic L cell FFA4 activity could increase PYY-Y_1 activity and thus reduce the I_{sc}. Assuming FFA4 also couples to $G_{\alpha q}$ in the neighbouring enterocytes, here activation of FFA4 may lead to the mobilisation of $[Ca^{2+}]_i$ via the PLC-PKC/IP_3 signalling pathway, to stimulate intermediate signalling cascades which increase Cl^- secretion (presumably via CFTR) thus raising mucosal I_{sc} (Berger et al., 1993; Billet and Hanrahan, 2013a; Billet et al., 2013b; Kunzelmann and Mehta, 2013). Therefore, L cell FFA4 activity may be counteracted by enterocyte FFA4 activity (Figure 4.4). In this way, in the presence of the FFA4 NAM, negative modulation of L cell FFA4 tonic activity would raise the I_{sc} and this could be counteracted by the action of the FFA4 NAM at the enterocyte FFA4 receptor i.e. a decrease in I_{sc} (Figure 4.4). As a result, no change in I_{sc} would be

observed upon addition of the FFA4 NAM and this could appear as a lack of FFA4 tone, as observed in this thesis.

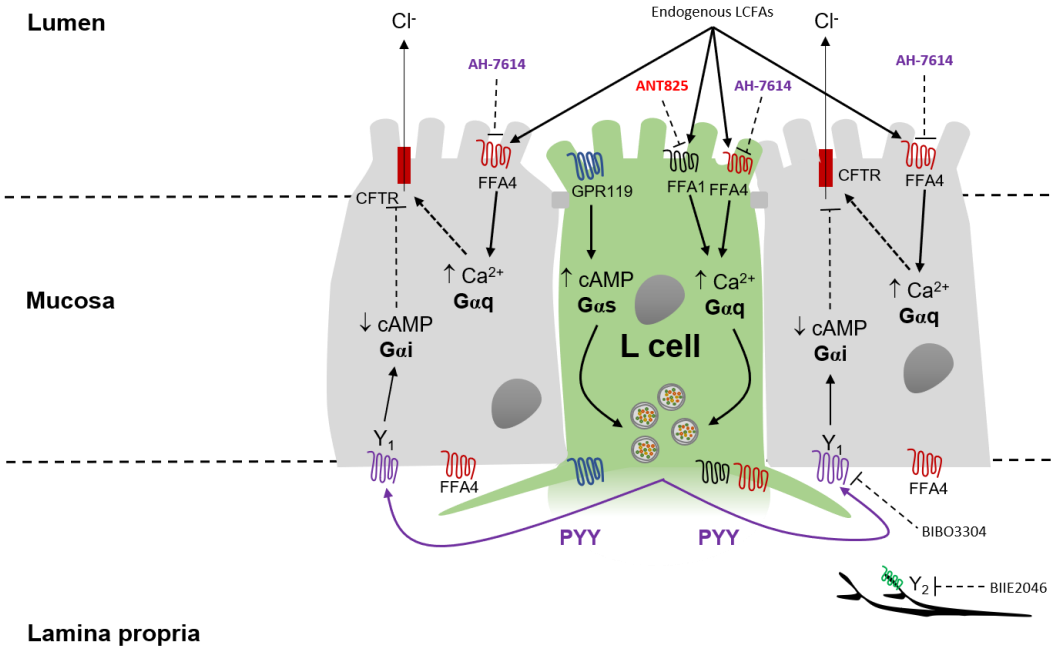


Figure 4.4: Proposed model of FFA1 and possible FFA4 tonic activity. Endogenous LCFAs activate FFA1 causing L cell PYY release, which activates epithelial Y_1 receptors in a paracrine manner. This causes inhibition of anion secretion. In the presence of a FFA1 antagonist, ANT825 the basal I_{sc} increased, indicating the FFA1 receptor was tonically active. The FFA4 receptor is widely expressed in enterocytes and enteroendocrine L cells. Thus, activation of FFA4 in L cells results in inhibition of anion secretion via a PYY-Y_1 mechanism, whereas activation of FFA4 in the enterocytes stimulates anion secretion. Y_1 and Y_2 receptors were blocked pharmacologically with the competitive Y_1 (BIB03304) and Y_2 (BIIE2046) antagonists, respectively.

The combination of the FFA4 NAM and FFA1 antagonist decreased basal I_{sc} (but this was not significant) and eliminated the FFA1 tone that was previously observed in the presence of ANT825 alone. Notably, L cells are outnumbered by the population of enterocytes in each mucosal preparation. In this way, the activity of the FFA4 NAM

in the surrounding enterocytes (i.e. decreasing basal I_{sc}) may counteract the increase in basal I_{sc} attributed to FFA1 tonic activity (Figure 4.4). As a result, an overall decrease in basal I_{sc} may be observed upon application of the FFA4 NAM in combination with the FFA1 antagonist, as observed in this PhD study. To investigate this hypothesis, the Y_1 and Y_2 receptors were selectively blocked with competitive Y antagonists, which eliminated the PYY-Y_1/Y_2 signalling attributed to L cell tonic activity (Figure 4.4). This allowed the effect of the FFA4 NAM to be further assessed specifically in enterocytes. Firstly, FFA1 tonic activity was abolished in the presence of the Y antagonists, and thus FFA1 tone was PYY Y_1/Y_2-mediated. Secondly, in the presence of both Y antagonists, the FFA4 NAM, AH-7614 significantly decreased the I_{sc}, as the consequence of L cell tonic activity which previously counteracted this response, was abolished. Furthermore, a significant decrease in I_{sc} to ANT825 + AH-7614 was observed in the presence of Y receptor blockade, further confirming FFA4 enterocyte activity was limited by L cell tonic activity. Taken together, FFA4 is expressed in enterocytes and L cells where it appears to be tonically active. Notably, the AZ FFA4 agonists did not reveal any indication of an FFA4-mediated enterocyte secretory mechanism. Thus, the FFA4 anti-secretory responses may be limited by a small secretory component that is not immediately evident in descending colon mucosa.

4.7 *FFA1, FFA4 and GPR119 responses were glucose-sensitive*

For the first time, selective activation of FFA1 and FFA4 in descending colon mucosa was shown to be glucose-sensitive. Additionally, GPR119 agonism was also glucose-sensitive, as shown previously. These findings were consistent with previous glucose-sensitivity observed in investigations of L cell-specific activation of GPR119 (Cox et al., 2010; Patel et al., 2014; Tough et al., 2018b), MC_4 (Panaro et al., 2014), the calcium-sensing receptor (Joshi et al., 2013) and FFA2 (Forbes et al., 2015). Furthermore, PYY responses were glucose-insensitive, and therefore enterocyte Y_1 receptors were able to respond to PYY, in the absence of glucose as observed in previous studies (Cox et al., 2010; Joshi et al., 2013; Panaro et al., 2014; Patel et al., 2014; Forbes et al., 2015; Tough et al., 2018b). In this way, the presence of glucose regulates L cell FFA1, FFA4 and GPR119 agonism, but does not affect downstream PYY-Y_1 signalling. As a result, this signalling pathway should only occur in the presence of luminal postprandial nutrients, and thus could protect against

hypoglycaemia *in vivo*, a disadvantage of current T2DM anti-diabetics on the market, namely sulphonylureas and insulin.

FFA1, FFA4 and GPR119 receptors were considered clinically favourable targets as they were not only co-expressed in GI L cells, but also differentially expressed in the pancreas. FFA1 and GPR119 were identified in pancreatic β-cells (Itoh et al., 2003; Chu et al., 2007) and FFA4 was recently discovered in somatostatin-containing delta cells (Stone et al., 2014). Thus, released L cell GLP-1 could prompt insulin release via GLP-1R on pancreatic β cells. Additionally, in the pancreas, a FFA1 or GPR119 agonist could simultaneously directly elevate insulin release postprandially. While FFA4 agonism inhibits glucose-stimulated somatostatin-14 secretion from pancreatic delta cells in murine islets of Langerhans, a mechanism that was $G_{\alpha i}$-coupled attributed to its pertussis-toxin sensitivity (Stone et al., 2014). Physiologically, somatostatin inhibits β cell insulin secretion. Therefore, FFA4 activation ($G_{\alpha i}$) in delta cells inhibits somatostatin release, which could result in prolongation of insulin secretion. Certainly, the LCFA, DHA potentiated insulin secretion in murine islets, and this functional effect was lost in islets isolated from $GPR120^{-/-}$ mice (Stone et al., 2014). This combination of indirect and direct FFA1, FFA4 and GPR119 effects could improve glucose tolerance with minimal risk of hypoglycaemia. Indeed, the FFA1 agonist that progressed to Phase III clinical trials, TAK-875, improved glucose tolerance and presented with a low risk of hypoglycaemia, in Japanese patients with T2DM (Kaku et al., 2015). This PhD study confirmed the glucose-sensitivity of TAK-875 in native mouse colonic tissue *in vitro.*

A lack of glucose-sensitivity has been displayed in primary canine L cells (Damholt et al., 1998) and fetal rat intestinal L cells (Roberge and Brubaker, 1991). These findings questioned the glucose responsiveness of the L cell in these species. Several studies in GLUTags (Reimann and Gribble, 2002; Gribble et al., 2003), primary transgenic mouse L cell cultures (Reimann et al., 2008), isolated perfused rat colon (Plaisancié et al., 1995) and in humans *in vivo* (Herrmann et al., 1995) have shown glucose triggers GLP-1 secretion. Initial investigations into the glucose-sensitivity of GLP-1 release in GLUTags has demonstrated that a high glucose-sensitivity in the range of 0 - 5 mM exists in this cell line (Reimann and Gribble, 2002; Gribble et al., 2003). A high glucose-sensitivity was also displayed in primary small intestine

cultures (EC_{50} = 4 mM) and colonic cultures (EC_{50} = 0.7 mM) (Reimann et al., 2008). As a result, GLP-1 release appeared glucose-sensitive and low millimolar glucose concentrations were required to elicit GLP-1 secretion. Controversially, in a recent study GPR119 agonism appeared to be glucose-independent in primary mouse colonic cultures and *in vivo* (Lan et al., 2012). Notably, Lan *et al.* (2012) utilised two independent GPR119 agonists, the first named B3 was tested in primary colonic cultures and the second GPR119 agonist, MBX-2982 was used *in vivo*. Firstly, ablation of GLP-1 secretion to B3 was not tested in primary colonic GPR119$^{-/-}$ cultures to confirm the glucose-insensitivity of its response was GPR119-mediated (Lan et al., 2012). Therefore, this functional effect of B3 may have been an off-target compound response. Secondly, the GPR119 agonist, MBX-2982 increased GLP-1 in the absence of glucose after an overnight fast *in vivo* (Lan et al., 2012). Notably, luminal (Kellett and Brot-Laroche, 2005) and plasma concentrations of glucose are around 5 mM after an overnight fast (Results, Figure 3.19). In this way, after an overnight fast, the glucose concentration is still adequate to cause L cell GLP-1 release, as the L cell glucose-sensitivity lies within a low millimolar range (Reimann and Gribble, 2002; Gribble et al., 2003; Reimann et al., 2008). In this way, GPR119-mediated (MBX-2982) GLP-1 secretion would be observed in the presence of "no glucose" in C57BL/6 mice. In another study, Lan *et al.* (2009) demonstrated a glucose-load enhanced GLP-1 secretion *in vivo* and this effect was eliminated in GPR119$^{-/-}$ mice (Lan et al., 2009). This further confirmed GPR119 responses were glucose-responsive, as shown previously (Chu et al., 2008; Cox et al., 2010; Patel et al., 2014; Tough et al., 2018b).

The glucose-sensitivity of the L cell has been attributed to the glucose-sensing machinery, SGLT1 and GLUT2, both of which are highly expressed in mouse primary cultured intestinal/colonic cells. Initial investigations of the low capacity/high affinity Na^+ glucose co-transporter, SGLT1 in these primary cultures revealed SGLT1 was widely expressed from the mid-intestine to the colon. The expression of the high capacity/low affinity facilitative glucose transporter, GLUT2 was high in the proximal GI tract and decreased along the length of the gut (Reimann et al., 2008). In the mouse, SGLT1 has been suggested as the predominant glucose absorption transporter in the GI tract (Wright et al., 2011).

4.7.1 L cell glucose-sensitivity machinery: SGLT1 and GLUT2 and their involvement in FFA1, FFA4 and GPR119 agonism

On basal I_{sc}, the combination of the SGLT1 inhibitor, phloridzin and the GLUT2 inhibitor, phloretin induced a time-dependent biphasic response. Firstly, the intake of Na^+ and glucose via apical SGLT1 increases I_{sc}. Thus, the presence of phloridzin revealed a decrease in I_{sc}, as observed previously (Cox et al., 2010; Joshi et al., 2013; Patel et al., 2014; Forbes et al., 2015; Panaro et al., 2014; Tough et al., 2018b). Secondly, uptake of glucose from the basolateral mucosal surface via GLUT2 decreases the I_{sc} and thus the GLUT2 inhibitor, phloretin is expected to increase the basal I_{sc}. In this way, the first component of the biphasic response observed under basal conditions was attributed to phloridzin and the second component to phloretin.

In this PhD study, blockade of SGLT1 and GLUT2 with phloridzin and phloretin respectively, abolished FFA1, FFA4 and GPR119 agonism. This implicated a critical requirement of SGLT1 and GLUT2 in FFA1, FFA4 and GPR119 L cell signalling. In the mouse, SGLT1 and GLUT2 are both expressed in intestinal EECs and the surrounding enterocytes. SGLT1 is trafficked to the luminal membrane (Reimann et al., 2008) and GLUT2 is located mainly in the basolateral membrane, but has also been identified in the apical membrane in mouse and rat (Gorboulev et al., 2012; Mace et al., 2012). The facilitative transporter GLUT2 mediates a minor role in small intestinal glucose absorption when the luminal glucose concentration levels are low (i.e. during fasting periods) (Kellett and Brot-Laroche, 2005). When the glucose concentration is low, GLUT2 is expressed minimally in the apical membrane. As the luminal glucose concentration rises, apical GLUT2 expression is upregulated, under the control of SGLT1, a mechanism that is eliminated in $SGLT1^{-/-}$ mice (Gorboulev et al., 2012) (Figure 4.5). A low level of GLUT2 expression in the apical membrane during fasting periods prevents glucose transport down its concentration gradient, from the plasma into the lumen via GLUT2. In this way, minimal apical GLUT2 expression provides a protective mechanism to prevent unnecessary elimination of glucose during periods of low glucose intake, and thus GLUT2 regulation is an essential glucose transporter to maintain glucose homeostasis.

SGLT1 and GLUT2 are critical regulators of incretin secretion. Loss of SGLT1 abolished the GLP-1 and GIP functional responses to an intragastric gavage of glucose in mice, demonstrating an important role of SGLT1 in L and K cell incretin release

(Röder et al., 2014). In another study, a significant loss of GLP-1 release to a glucose load was revealed in GLUT2$^{-/-}$ mice (Cani et al., 2007), clearly linking GLUT2 to the regulation of incretin secretion. Similarly, GLUT2 is essential for approximately 55 % of incretin secretion in L and K cells in rats (Mace et al., 2012). In this way, the glucose-sensing machinery, SGLT1 and GLUT2 of enteroendocrine L cells ensures peptide secretion is glucose-sensitive. Approximately >80 % of D-glucose absorption in the luminal membrane occurs primarily via the Na^+ glucose-cotransporter, SGLT1 in mice (Gorboulev et al., 2012). This was confirmed by Röder *et al.* (2014) as approximately 80 % loss of glucose uptake was demonstrated in SGLT1$^{-/-}$ mice. As GLUT2 is trafficked in high glucose to the luminal membrane to ensure luminal and plasma glucose equilibrates, it is unlikely apical GLUT2 plays a major role in this PhD study as FFA1, FFA4 and GPR119 signalling was assessed in the presence of 11.1 mM glucose, a concentration similar to the measured plasma glucose concentration before a 16 h overnight fast (at ~ 5.30 pm i.e. mice were satiated and resting) (Results, Figure 3.19). Therefore, in this thesis, the glucose-sensitivity of FFA1, FFA4 and GPR119 was most likely primarily mediated by apical SGLT1 and basolateral GLUT2, however a role for apically-expressed GLUT2 cannot be ruled out.

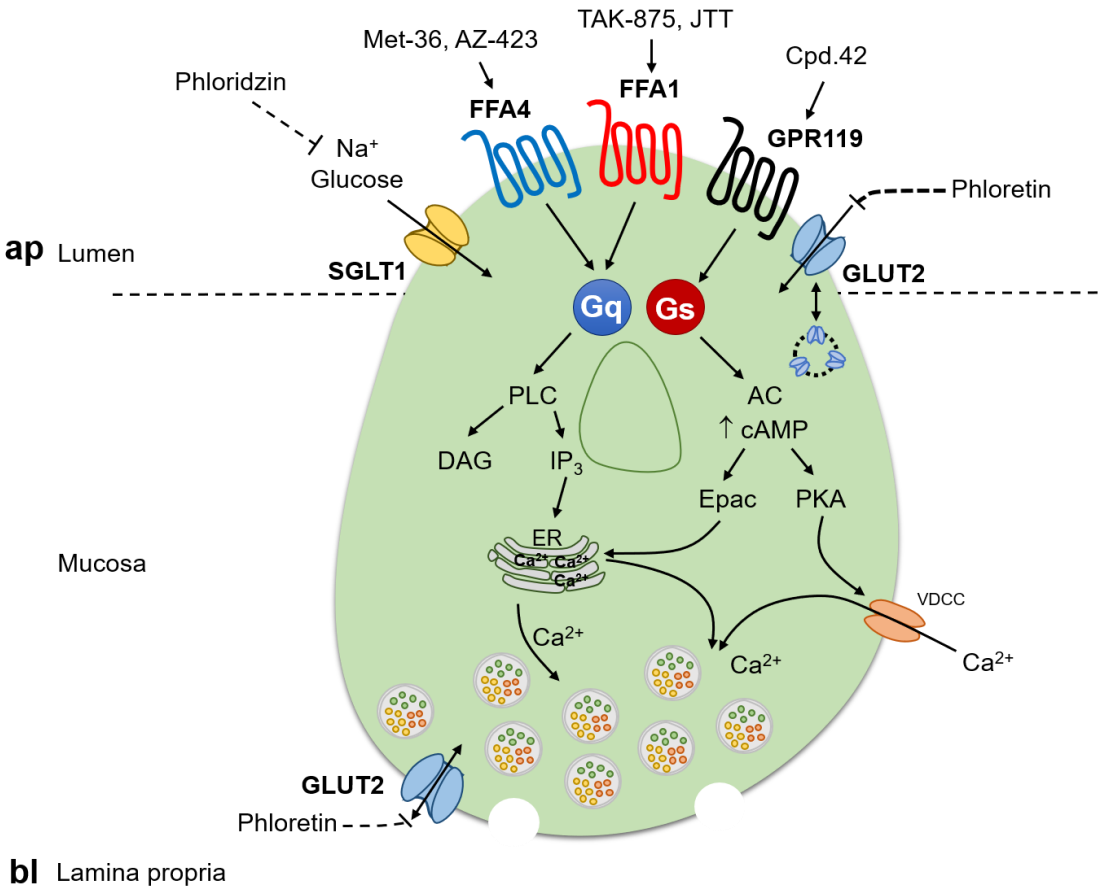


Figure 4.5: FFA1, FFA4 and GPR119 glucose-sensitivity is mediated via SGLT1 and GLUT2 in the L cell. Activation of FFA1, FFA4 and GPR119 with selective agonists (apical application) stimulated $G_{\alpha q}$ (FFA1 and FFA4) and $G_{\alpha s}$ (GPR119) signalling, only in the presence of apical glucose. Furthermore, in the presence of glucose, FFA1, FFA4 and GPR119 responses are eliminated in the presence of SGLT1 inhibitor, phloridzin and the GLUT2 blocker, phloretin. SGLT1 is the predominant glucose-transporter of the L cell and apical GLUT2 is expressed at low levels during fasting periods and is upregulated during high glucose, to equilibrate glucose levels in the plasma and lumen.

4.8 *Co-agonism of FFA1, FFA4 and GPR119 was additive, not synergistic in the descending colon*

Physiologically after a meal, triglycerides are hydrolysed by intestinal pancreatic lipase yielding, 2-MAG and FFAs, endogenous activators of enteroendocrine L cell

GPR119 and FFA1/FFA4, respectively (Figure 1.2). In this way, postprandial concomitant activation of FFA1, FFA4 and GPR119 may act synergistically to enhance peptide release, particularly PYY release that could be measured indirectly via the Y_1 epithelial response. To probe for possible synergistic effects between FFA1, FFA4 or GPR119 activation, highly selective synthetic agonists (at EC_{80} concentrations) were chosen to stimulate two or more receptors (triple receptor agonism discussed in section 4.10) at any one time, in descending colon mucosa. The combined agonist responses were compared with the responses to single agonist application. Notably, combined agonists were added to the mucosa in a single application to mimic experiments carried out in murine colonic primary culture preparations (Ekberg et al., 2016; Hauge et al., 2017).

In this thesis, all the responses to the following co-agonism combinations of selective FFA1, FFA4 and GPR119 agonists: FFA1 + FFA4, FFA1 + GPR119 and FFA4 + GPR119, were additive but not synergistic, when compared with responses exhibited by single agonism. This demonstrated that the combined agonism of two independent receptors increased the capability of the L cell to secrete PYY, as measured by the larger anti-secretory response. Furthermore, the delayed maxima of the combined responses to GPR119 + FFA1 agonism and GPR119 + FFA4 agonism compared to the maxima of single agonism may be attributed to the integration of intracellular signalling pathways in the L cell and/or the surrounding enterocytes, as a consequence of coincident $G_{\alpha s}$-sustained (GPR119) and $G_{\alpha q}$-transient (FFA1 or FFA4) L cell activation. The additive effects of combined agonism were consistent with the additive findings observed by Hauge *et al.* (2017) but were at odds with the synergistic effects observed by Ekberg *et al.* (2016). In murine colonic cultures prepared from C57BL/6 mice, the synergistic effects of selective potent agonists were assessed by measuring GLP-1 secretion over a 3 h agonist incubation period, in the presence of 10 mM glucose. A synergistic response was defined as a response that was greater than an additive effect. Ekberg *et al.* (2016) observed synergistic GLP-1 secretion when utilising a low concentration of a GPR119 agonist (AR231453, 10^{-9} M) along with a higher concentration of a FFA1 agonist (TAK-875, 10^{-5} or 10^{-6} M). Furthermore, an additive, not a synergistic effect was revealed when combining the GPR119 agonist (AR231453, 10^{-9} M) with a FFA4 agonist (Metabolex-209, 10^{-5} or 10^{-6} M) (Ekberg et al., 2016), as seen in the current study. The absence of a synergistic effect of the

combined FFA4 agonist (Metabolex-209) and GPR119 agonist to stimulate GLP-1 secretion, confirms the minor role of the FFA4 receptor in GLP-1 release. Later in a separate study, the combination of a GPR119 agonist (AR246881, 10^{-9} M) and a FFA1 agonist (MK-2035, 10^{-8} – 10^{-7} M) in murine primary colonic cultures was additive and not synergistic (Hauge et al., 2017). This was most likely attributed to the utilisation of a low concentration (nM range) of the FFA1 agonist, in comparison to the higher concentration (µM range) used by Ekberg *et al.* (2016). It would appear certainly in these two studies, that a low concentration (nM range) of a GPR119 agonist (coupled to $G_{\alpha s}$) and a higher concentration (µM range) of a FFA1 agonist (coupled to $G_{\alpha q}$) is required to elicit synergistic GLP-1 release.

In the present study, the concentration of the FFA1 agonist, TAK-875 was identical to the TAK-875 concentration (1 µM) utilised by Ekberg *et al.* (2016). Thus, the absence of an observed synergistic effect in the present study may be attributed to the use of a rather high concentration ($EC_{80,}$ nM - µM) of a GPR119 agonist, Cpd.16 compared to the lower concentration of a GPR119 agonist (nM range) used in recent published investigations (Ekberg et al., 2016; Hauge et al., 2017). Notably, differences in assay preparations (i.e. Ussing chamber mouse mucosal sheets versus mouse primary colonic cultures) may contribute to the observed lack of synergy in the current study. While both assays utilised the mouse colon in the presence of glucose, the present study specifically investigated mechanisms in the descending colon, in single mucosal preparations (exposed area, 0.14 cm^2), whereas murine colonic cultures were created by mincing the entire mouse colon (Ekberg et al., 2016; Psichas et al., 2017). In murine colonic cultures, active GLP-1 release was measured over a 3 h period in primary L cell cultures, whereas Ussing chamber mucosal responses to agonism or co-agonism were monitored over 20 mins. One could argue that the mucosal responses were measured in more of an acute setting in native tissue. Furthermore, within this 20 min period, mucosal responses represented a downstream PYY-Y_1 paracrine mechanism (in the absence of DPPIV-inhibitor) and was not a direct measure of GLP-1 release from L cells. Hence, PYY is likely to undergo DPPIV hydrolysis before it can bind enterocyte Y_1 receptors, limiting the I_{sc} recording of the actual PYY-Y_1 response. As a result, if co-agonism was indeed synergistic, PYY degradation limits this observation. Moreover, co-agonism investigations in murine primary L cell cultures measured GLP-1 secretion, and in this present study a GLP-1 component was

not observed in FFA1, FFA4 or GPR119 agonism. Therefore, this PhD study cannot rule out the fact that differences between the described additive responses and synergistic effects observed by Ekberg *et al.* (2016) may be attributed to the recent discovery of GLP-1 only and PYY only segregated L cells (Cho et al., 2015). Furthermore, GLP-1 and PYY storage has also been identified in separate vesicles in the same cell (Cho et al., 2014). In this way, activation of the L cell with potent and selective FFA1, FFA4 or GPR119 agonists could lead to differential L cell GLP-1 and PYY release, which may also be dependent on the intestinal region under investigation.

4.9 The dietary FFA, pinolenic acid was a dual FFA1 and FFA4 agonist

The dietary FFA, pinolenic acid constitutes 15-20 % of PNO (Christiansen et al., 2015) and administration of PNO has been associated with a reduction in food intake (Hughes et al., 2008; Pasman et al., 2008) and weight gain (Le et al., 2012; Park et al., 2013), and an increase in energy expenditure (Hughes et al., 2008). Pasman *et al.* (2008) observed a 25 % rise in GLP-1 and a 60 % increase in CCK-8 in overweight post-menopausal woman after administration of PNO, compared to the control group. The rise in these two peptide hormones was thought to contribute to appetite suppression (Pasman et al., 2008). The FFA1 and FFA4 receptors are expressed in GLP-1 containing L cells (Hirasawa et al., 2005; Edfalk et al., 2008) and CCK-containing I cells (Liou et al., 2011; Sykaras et al., 2012). Recently, pinolenic acid was described as a selective dual FFA1 and FFA4 agonist in HEK293 cells (Christiansen et al., 2015). In this PhD study, the function and pharmacology of pinolenic acid was tested in mouse native descending colon for the first time. Here, the potency and efficacy of pinolenic acid resembled that of the commercially available dual agonist, GW9508. Moreover, the potency in native tissue was consistent with the potencies exhibited in HEK293 cells expressing mouse FFA1 or FFA4, in a Ca^{2+} mobilisation assay (Christiansen et al., 2015). Christiansen *et al.* (2015) demonstrated pinolenic acid was similarly potent at the mouse FFA1 and FFA4 receptors in a Ca^{2+} mobilisation study, with EC_{50} values of 2.8 µM and 3.6 µM, respectively (Christiansen et al., 2015). In the current study, like GW9508, pinolenic acid was selective for both the FFA1 and FFA4 receptors, demonstrating dual agonism in native tissue. The residual pinolenic acid mucosal response in the presence of the combined FFA1 antagonist and FFA4 NAM was attributed to the NAM properties of

AH-7614 (Watterson et al., 2017). The pinolenic acid response was abolished in the presence of the competitive Y_1 and Y_2 antagonists, BIBO3304 and BIIE2046, respectively. Therefore, L cell derived PYY acts via Y_1 and presumably Y_2, underpinning the pinolenic acid mucosal response. Physiologically, the released PYY could potentially reduce GI motility (Pironi et al., 1993; Lin et al., 1996; Lin et al., 2000; Tough et al., 2011), increase central satiety ($PYY_{(3-36)}$) and reduce food intake and weight gain. Thus, pinolenic acid may contribute to the beneficial effects of PNO administration described earlier in humans and rodents (Pasman et al., 2008; Le et al., 2012; Park et al., 2013).

4.10 Triple co-agonism of FFA1, FFA4 and GPR119 was not additive and potentially led to Y_1 receptor desensitisation

In the presence of the triglyceride, olive oil, Ekberg *et al.* (2016) utilised various knockout mice ($FFA1^{-/-}$, $FFA4^{-/-}$, $GPR119^{-/-}$ and double $FFA1/FFA4^{-/-}$) to shown FFA1, FFA4 and GPR119 all contributed to GLP-1 release, but to different extents *in vivo*. Ekberg *et al*. (2016) demonstrated that the GPR119 receptor and the FFA1 receptor had a similar importance for GLP-1/GIP release after lipid ingestion, while FFA4 had a relatively minor contribution (Ekberg et al., 2016). To date, this PhD study is the first to show concomitant agonism of L cell FFA1, FFA4 and GPR119 with potent and selective synthetic agonists, in native mouse descending colon mucosal preparations *in vitro*. These experiments display a more accurate representation of postprandial L cell activation compared to co-agonism. Triple receptor agonism was assessed utilising EC_{80} concentrations of the dual FFA1 and FFA4 agonist, pinolenic acid in combination with a full GPR119 agonist, PSN632408 or the AZ selective GPR119 agonist, Cpd.16. Triple receptor agonism was hypothesised to result in an additive mucosal anti-secretory response, presumably larger than the co-addition mucosal responses. However, the combined response to pinolenic acid with each GPR119 agonist was not additive. Moreover, PSN632408 L cell signalling appeared limited when this agonist was added in combination with the dual agonist, pinolenic acid. The concentration of PSN632408 was potentially too high, and a lower concentration (in the nM range) should ideally be assessed in accordance with previous studies (Ekberg et al., 2016; Hauge et al., 2017), to observe potential additive or synergistic effects.

Notably, while L cell triple receptor agonism appeared 'blunted' it is possible that PYY release was additive. However, mucosal anti-secretory responses are a measurement of the consequent paracrine effects of PYY release, involving further enterocyte downstream signalling. In this way, a large increase in PYY release evoked by triple receptor agonism could potentially cause desensitisation and internalisation of the finite population of Y_1 receptors in the surrounding enterocytes, in each mucosal preparation. The neighbouring enterocyte Y_1 receptors might be less responsive to the large increase in PYY and therefore conceal any potential additive effects (Figure 4.6). Indeed, Holliday and Cox (2003) demonstrated in colonic carcinoma epithelial layers that as the concentration of exogenous PYY increased (3 nM – 1 μM), the PYY response became more transient and this was possibly a consequence of Y_1 receptor desensitisation (Holliday and Cox, 2003; Holliday et al., 2005). Furthermore, the Y_1 receptor underwent agonist (NPY)-stimulated phosphorylation and the involvement of the G-protein coupled receptor kinase 2 (GRK2) was critical in this process. Phosphorylation of the Y_1 receptor by GRK2 was required for β-arrestin 2 recruitment and this signalling pathway most likely underpinned Y_1 receptor desensitisation. Furthermore, NPY (100 nM) induced rapid (within 5 min) internalisation of Y_1 receptors via clathrin-dependent endocytosis, and this was inhibited by the pretreatment of the competitive Y_1 antagonist, BIBO3304, in HEK293 cells (Holliday et al., 2005). In another study, the Y_1 receptor was also shown to rapidly internalise upon agonist stimulation in HEK293 cells, via clathrin-dependent endocytosis (Gicquiaux et al., 2002). Gicquiaux *et al.* (2002) revealed submembrane internalisation occurred within 5 min (Gicquiaux et al., 2002; Pheng et al., 2003) and endocytosed receptors recycled back to the submembrane region slowly, but within 60 min (Gicquiaux et al., 2002). More recently, using super-resolution imaging, NPY (100 nM) was shown to induce rapid clathrin-dependent Y_1 internalisation (within 5 min) in HEK293 cells (Kempf et al., 2015). Thus, as the Y_1 receptor undergoes rapid desensitisation and internalisation, it is possible that the increase in PYY release after triple receptor agonism (pinolenic acid + PSN632408) led to Y_1 desensitisation and presumably subsequent internalisation. As a result, the anti-secretory response would appear 'blunted' despite the high concentration of released PYY.

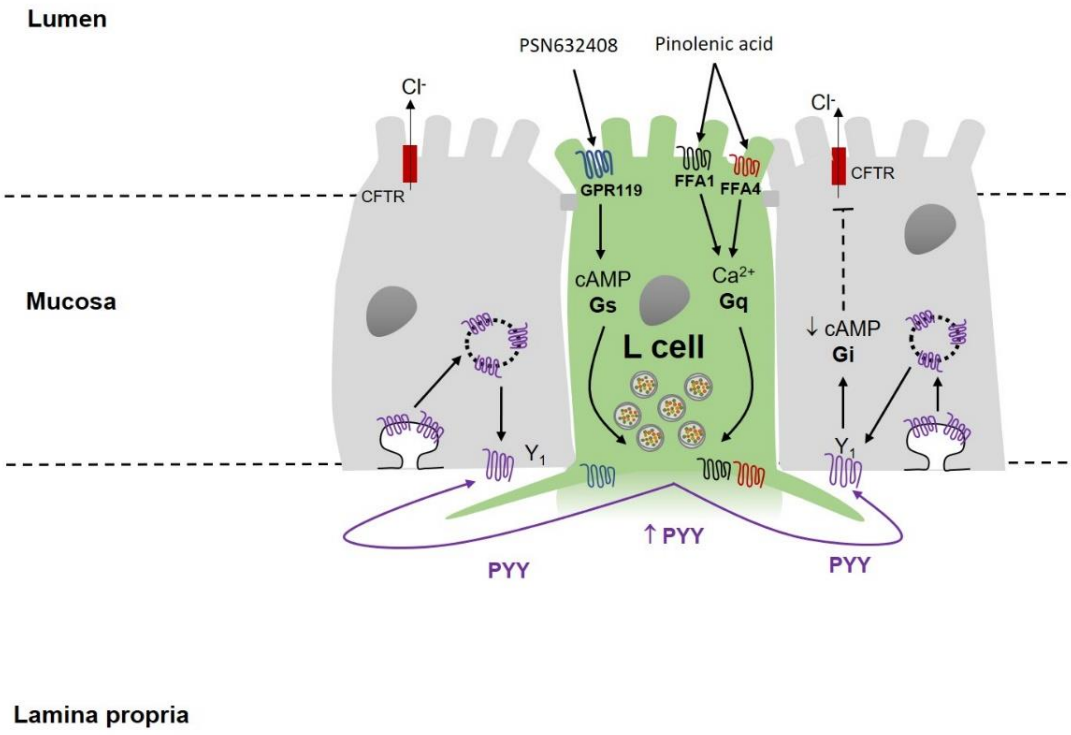


Figure 4.6: FFA1, FFA4 and GPR119 agonism in the L cell. The GPR119 agonist, PSN632408 elicits a large PYY-Y_1($G_{\alpha i}$) anti-secretory response. However, additional application of the dual FFA1 and FFA4 agonist, pinolenic acid limits PSN632408-induced L cell signalling, presumably as a consequence of a large increase in released PYY, which leads to PYY-Y_1 desensitisation and internalisation via clathrin-dependent endocytosis. The endocytosed Y_1 receptors are recycled back slowly to the plasma membrane.

Taken together, in this thesis, the combination of two agonists (utilising an EC_{80} concentration) resulted in additive responses. However, an appropriate low concentration (nM range) of a GPR119 agonist applied concomitantly with a high concentration (µM range) of a FFA1 agonist, appears to act synergistically to release L cell peptides (i.e. GLP-1) (Ekberg et al., 2016). Physiologically, a very low concentration of nutrients arrives in the distal intestine postprandially, as most nutrients are primarily absorbed and sensed in the proximal intestine. The concentration of nutrients that arrive in the distal intestine may be low enough (nM - µM, Ekberg et al., 2016) to elicit co-agonism and synergistic GLP-1 and presumably

PYY release, *in vivo.* In this way, these nutrients may act to prolong the slowing of transit and enhance satiety during fasting periods. Pathophysiological conditions that induce steatorrhoea (as described in section 4.3.3), increase the intraluminal lipid content in the colon and this enhances PYY release (Adrian et al.,1986). This rise in PYY levels may desensitise Y_1 signalling (Holliday and Cox, 2003), and may also cause internalisation of the Y_1 receptor (Holliday et al., 2005). Thus, unresponsive PYY-Y_1 signalling attributed to Y_1 desensitisation and internalisation, may contribute to the reason steatorrhoea is associated with more frequent and liquid stools i.e. diarrhoea (Ung et al., 2000), despite PYY being a known defender against diarrhoea (Playford et al., 1990).

4.11 FFA1 and FFA4 agonism slowed colonic motility in vitro

FFA1 (TUG424, TAK-875) and FFA4 (TUG891, Met-36) agonists attenuated colonic motility *in vitro* to a similar degree, as seen previously with MC_4 (Panaro et al., 2014) and GPR119 agonism (Tough et al., 2018b). These agonists decreased colonic motility presumably via the release of PYY, a known mediator of the colonic brake (Lin et al., 1996; Tough et al., 2011). Pretreatment with the FFA1 antagonist, GW1100 revealed basal colonic mucosal tone (as seen in Ussing chamber studies), indicative of endogenous FFA1 activity. In the presence of the FFA1 antagonist GW1100, the colonic brake induced by the FFA1 agonist, TUG424 was disinhibited and therefore colonic transit increased, whereas transit induced by the FFA4 agonist, TUG891 remained unaffected, demonstrating the selectivity of GW1100 for the FFA1 receptor.

4.11.1 FFA4 not FFA1 agonism slowed colonic motility in vivo

As the FFA1 agonist, TAK-875 and the FFA4 agonist, Met-36 slowed transit *in vitro,* the translation of these effects was investigated *in vivo*. Following the overnight fast, plasma glucose levels were reduced to 5 mM, a concentration similarly observed by Han *et al.* (2008) after an overnight fast in C57BL/6J mice (6 mM). However, when FFA1 and FFA4 signalling was assessed in the presence of 5 mM glucose, mucosal responses were no different when compared with signalling in the presence of 11.1 mM glucose, confirming the reduction in plasma glucose after an overnight fast does not affect FFA1 or FFA4 signalling. Reimann and Gribble (2002) demonstrated that GLP-1 secretion was elevated as glucose increased from 0 - 5 mM in GLUTags, but

secretion plateaued as the glucose concentration incrementally increased from 5 - 25 mM. This revealed a low micromolar glucose-sensitivity of GLP-1 secretion (Reimann and Gribble, 2002), which was also shown in mouse L cell primary cultures (Reimann et al., 2008). Indeed, this was consistent with the findings in this thesis, as an incremental increase in the glucose concentration from 5 to 25 mM did not affect FFA1 and FFA4 agonism.

Met-36 significantly slowed colonic transit presumably via PYY, whereas TAK-875 failed to show significant inhibition of bead colonic transit (when administered orally or via i.p.) or UGIT (i.p only). This apparent lack of efficacy may be due to the administration of TAK-875 as a suspension. In the *in vitro* transit studies, the isolated colon is severed from the CNS and TAK-875 acts directly upon colonic FFA1 receptors. In the *in vivo* study, it's possible that the ability of TAK-875 to release PYY is not sufficient to alter colonic motility, with the added complication of modulatory CNS pathways. As ANT825 had no effect on colonic or upper intestinal transit, the observed FFA1 tonic activity *in vitro* (Ussing chamber studies) was presumably not sufficient to result in an increase in transit *in vivo*. The positive control, loperamide HCL, significantly slowed colonic and UGIT, as shown previously (Myagmarjalbuu et al., 2013). Notably, there are concerns in the literature that isoflurane interferes with GI transit. In 2005, Torjman and colleagues reported that 6 min exposure to isoflurane in rats slows UGIT by 50 % (Torjman et al., 2005). In this PhD study, it is important to note mice received a 2 min exposure of isoflurane 1 h after the drug, vehicle or positive control were administered, followed by bead insertion and excretion. Therefore, this brief exposure of isoflurane in the protocol (at the time of maximal drug exposure) should have minimal effect on colonic motility. Furthermore, there were no differences in colonic transit rates between different vehicle controls (*po* vs i.p.) in this study, compared with those in a previous investigation (Forbes et al., 2012).

4.11.2 The FFA4 agonist, Met-36 increased motility in the upper intestine

Unexpectedly, Met-36 was pro-motile in the upper intestine *in vivo,* when utilising the same dose that slowed transit in the colon. In the upper intestine, FFA4 is expressed by I cells, K cells and L cells, causing release of CCK, GIP and PYY respectively, via $G_{\alpha q}$-signalling (Hirasawa et al., 2005; Tanaka et al., 2008; Sykaras et al., 2012; Iwasaki

et al., 2015). FFA4 has also been identified in gastric ghrelin and somatostatin cells, however FFA4 signals via $G_{\alpha i}$ to inhibit peptide release in these cells (Engelstoft et al. 2013, Egerod et al. 2015). GIP (Ogawa et al., 2011) and PYY (Lin et al., 1996) slow small intestinal transit, while CCK increases upper intestinal transit in mice (Wang et al., 2004). In this way, activation of I cell FFA4 may have contributed to the observed increase in transit, and thus antagonising the CCK-1 and CCK-2 receptors should abolish this effect. More recently, RT-PCR revealed FFA4 in duodenal and colonic enterochromaffin cells (ECCs), which are responsible for secreting 5-HT (Martin et al., 2017). These ECCs were recently revealed as a heterogenous population in the mouse. In the upper intestine, particularly the mouse duodenum and jejunum, almost all of the 5-HT was found colocalised with CCK (50 – 60 %) or CCK with secretin (40 – 50 %) (Reynaud et al., 2016). To date, there are no studies that have investigated FFA4 G-protein signalling mechanisms in ECCs. In isolated guinea pig ileum, luminal perfusion of the MCFA, decanoic acid (1 mM) evoked propulsion and segmentation via release of 5-HT and CCK. This was inhibited by antagonists of the 5-HT_3, 5-HT_4, CCK-1 and CCK-2 receptors, demonstrating critical roles for these four receptors in fatty acid-induced propulsion and segmentation (Ellis et al., 2013). As the FFA4 receptor is expressed in duodenal enteroendocrine I cells (Sykaras et al., 2012) and ECCs (Martin et al., 2017), activation of FFA4 here could prompt CCK and 5-HT secretion, to enhance motility and segmentation (Diwakarla et al., 2017). Taken together, this suggests that in this thesis, Met-36 potentially activated FFA4 on ECCs and I cells to evoke 5-HT and CCK release, which may have increased UGIT. This stimulation of UGIT may aid duodenal digestion and absorption. Additionally, CCK is known to stimulate distal L cell PYY release, which slows motility and enhances nutrient absorption in the proximal intestine, via the ileal and colonic brakes (Lin et al., 1996; Lin et al., 2000). Notably, there was no indication of a 5-HT- or CCK-mediated component to the FFA4 mucosal response in the upper intestine (mid-ileum) *in vitro*, and thus this potential mechanism was only observed *in vivo*.

4.12 The prospects for future FFA1, FFA4 and GPR119 agonists in the pharmaceutical industry

The AZ FFA1, FFA4 and GPR119 selective agonists were synthesised to investigate the potential of the FFA1, FFA4 and GPR119 receptors as anti-diabetic therapeutic targets. These three receptors are expressed in the pancreas (Itoh et al., 2003; Chu et

al., 2007; Stone et al., 2014) and also in intestinal GLP-1 containing L cells (Hirasawa et al., 2005; Chu et al., 2008; Edfalk et al., 2008). Theoretically, due to their distinctive distribution, selective agonism could cause intestinal L cell peptide release, and FFA1 or GPR119 agonism might stimulate β cell insulin secretion, via a glucose-sensitive pathway and thus limit hypoglycaemia. Indeed, in the current study FFA1, FFA4 and GPR119 agonism was glucose-sensitive in L cells. This characteristic provides an advantage over some anti-diabetic therapeutics that cause hypoglycaemia, namely sulfonylureas and insulin.

Although several GPR119 and FFA1 agonists have been synthesised and progressed into clinical trials, only one has surpassed phase II clinical trials, the FFA1 agonist, TAK-875 (Kaku et al., 2015; Li et al., 2016; Ritter et al., 2016). The FFA1 agonist, JTT-851 (Japan Tobacco) has recently completed phase II studies, however no clinical outcomes have been disclosed (NCT01699737). Unfortunately, several agonists that have progressed to phase II clinical trials have been terminated, often without disclosure of the underlying reason. The GPR119 agonist, GSK1292263 that reached phase II clinical trials exhibited no safety issues, however no alterations in glucose, insulin or glucagon were observed when this agonist was administered alone or in combination with metformin. This clinical trial was terminated due to lack of GSK1292263 efficacy (Nunez et al., 2014). In the present study the novel AZ compounds were selective and potent but displayed low maximal responses in native mouse colonic mucosa, whereas the GPR119 agonists displayed partial activity in comparison to the commercial GPR119 agonist, PSN632408. The lack of efficacy of the AZ compounds may be attributed to potent β-arrestin recruitment and subsequent receptor desensitisation/internalisation, resulting in tachyphylaxis. Full agonists should be taken into consideration in future chemical optimisation studies, as they exhibit greater efficacy. However, full agonists are often accompanied by low potency, high lipophilicity and large molecular size, which limits compound absorption. Agonists that are highly lipophilic increase the potential for systemic permeation, CYP enzyme and hERG inhibition, and other unwanted side effects attributed to the peripheral and central expression (excluding the gut) of FFA1, FFA4 and GPR119. Recently, the expression of GPR119 was identified in skeletal myotubes and cardiac myoblasts *in vitro*. GPR119 agonism (PSN632408) induced detrimental fatty acid/glucose metabolism and oxidation in skeletal myotubes (Cornall et al.,

2013b), and downregulated genes involved in nutrient metabolism and inflammation states in H9c2 cardiac myoblasts (Cornall et al., 2012). To date, it remains unknown whether these unwanted GPR119 effects will translate into rodent and human studies *in vivo*. A luminal-restricted GPR119 agonist may increase the safety profile of a potential GPR119 candidate and minimise the adverse systemic effects. However, a luminal-restricted GPR119 agonist would limit direct β cell GPR119 signalling and therefore rely on L cell GPR119 activation alone to improve glucose tolerance. That said, for the first time β cell GPR119 agonism (AR231453) resulted in no difference in insulin, GLP-1 levels and glucose tolerance in selective β cell GPR119$^{-/-}$ and WT mice (Panaro et al., 2017). This suggested activation of β cell GPR119 does not mediate the beneficial effects of GPR119 agonism, and therefore these effects may be solely incretin-mediated. Thus, a potent orally available GPR119 agonist, which is restricted to the gut lumen and can substantially elevate L cell GLP-1 release, could increase the success rate of future GPR119 therapeutics. Whether this is also the case for the β cell FFA1 receptor has yet to be elucidated. The TAK-875 clinical trial was terminated due to liver failure (Kaku et al., 2015), which has been attributed to possible inhibition of liver bile transporters (Otieno et al., 2017; Wolenski et al., 2017). As FFA1 receptors are not expressed in hepatocytes, the liver toxicity may have been a specific TAK-875 compound effect, and therefore holds promise for future FFA1 clinical candidates. However, luminal-restricted FFA1 agonists should be considered in future investigations.

To date the high rate of attrition of FFA1 and GPR119 clinical candidates has been disappointing. This has been attributed to the lack of translatability between preclinical animal studies and human clinical trials. Additionally, potential variation in human and rodent enteroendocrine receptor expression and signalling variation may contribute to this attrition rate. In terms of the FFA1 agonist, TAK-875, while many *in vitro* studies in primary cultures demonstrated TAK-875 increased GLP-1 secretion (Hauge et al., 2015; Ekberg et al., 2016; Hauge et al., 2017), this functional effect was not observed in native colonic tissue in this PhD study, and also did not translate in human studies (Araki et al., 2012; Kaku et al., 2015). This questioned the ability of these agonists to stimulate GLP-1 release *in vivo*. Thus, there is a clear preclinical need for assays based in native tissue *in vitro*, before carrying out *in vivo* experiments. This approach might provide the best chance of observing an accurate and translatable

response *in vivo*. Another confounding factor may be the fact that agonism of FFA1, FFA4 or GPR119 may not result in substantial GLP-1 and PYY secretion, to mimic the high GLP-1/PYY plasma levels observed in individuals after bariatric surgery, which are thought to underlie the successful weight loss and improvement in glucose tolerance in these individuals (Miras and le Roux, 2013). Furthermore, there has been an increasing body of evidence over the length of this PhD to suggest the FFA4 receptor has a minor contribution to GLP-1 release (Xiong et al., 2013; Paulsen et al., 2014; Ekberg et al., 2016). Over the years, FFA4 has been identified in enterocytes and L cells. Thus, FFA4 is more broadly expressed in the intestine compared to the other L cell lipid receptors, GPR119 and FFA1. As a result, selectively targeting L cell FFA4 becomes problematic. The complexity increases as FFA4 couples to $G_{\alpha i}$ in other EEC types in the gut (Egerod et al., 2015; Koyama et al., 2016). In this way, agonists may possess stimulatory effects in one cell but counter inhibitory effects in another. To date, no FFA4 agonists have progressed to clinical trials. While the FFA4 receptor was presented as an ideal target for T2DM in 2005 (Hirasawa et al., 2005), today the FFA4 receptor is not a suitable candidate for T2DM.

Taken together, as co-agonism of FFA1 and GPR119 act synergistically to prompt GLP-1 secretion in mice (Hauge et al., 2015; Ekberg et al., 2016) and co-agonism of these receptors is additive in native descending colonic mucosa, future T2DM therapeutics should optimally target FFA1 and GPR119. Luminal-restricted FFA1 and GPR119 agonists that are potent, efficacious and improve glucose-tolerance while potentiating satiety, should be considered as future anti-T2DM and anti-obesity therapeutics.

4.13 Conclusions

Selective FFA1, FFA4 and GPR119 agonism potently inhibited mucosal anion secretion via a PYY-Y_1 paracrine mechanism. Mucosal responses were bi-directional, and therefore these receptors are most likely expressed on both the luminal and serosal domains of the L cell. The novel AZ agonists were selective and more potent than the commercially available agonists but induced low maximal responses. The AZ GPR119 agonists displayed partial activity in comparison to PSN632408, indicative of potential β-arrestin recruitment. This may have resulted in acute desensitisation

and might be a possible contributing factor to the failure of many GPR119 and FFA1 clinical candidates in the past. FFA1, FFA4 and GPR119 mucosal responses were greatest in the ileum, ascending colon and descending colon, respectively. Thus, these three receptors may have a major role to play in pathophysiological malnutrition states e.g. steatorrhoea. Furthermore, FFA1, FFA4 and GPR119 agonism was independent of enteric neurons, and therefore their responses were epithelial in origin. The glucose-sensitivity of FFA1, FFA4 and GPR119 agonism was mediated by SGLT1 and GLUT2, and may well provide protection against hypoglycaemia, a side effect of current T2DM therapeutics, namely sulphonylureas and insulin. Dual agonism was additive in native tissue, while the signalling to triple receptor agonism appeared limited, a possible consequence of Y_1 desensitisation.

The competitive Y_1 and Y_2 antagonists revealed Y_1 and Y_2 tone respectively, an indicator of tonic PYY release. Additionally, the FFA1 antagonists, ANT825 and GW1100 revealed tonic FFA1 activity. This suggested an endogenous ligand was activating the FFA1 receptor. The lack of FFA4 tone in the presence of the FFA4 NAM, AH-7614 may have been attributed to the broad expression of the FFA4 receptor in L cells and the surrounding enterocytes. Agonism of FFA1 and FFA4 inhibited colonic transit *in vitro,* and FFA4 agonism induced regional differences in transit *in vivo.* In the colon, FFA4 agonism slowed transit presumably via PYY, whereas in the upper GI tract FFA4 agonism was pro-motile and potentially involved 5-HT/CCK-containing ECCs in the mouse *in vivo*.

Taken together, FFA1 and GPR119 co-agonism may be beneficial therapeutically in T2DM and obesity in the form of potent luminal-restricted agonists, while the FFA4 receptor does not appear to be a suitable target for future anti-diabetic and anti-obesity therapeutics attributed to its widespread distribution along the length of the GI epithelium. Co-agonism more closely mimics the combinatorial activity of triglyceride metabolites in the diet. Therefore, a GPR119 agonist in combination with a FFA1 agonist may act synergistically to amplify hormone release, and thus improve glucose tolerance and enhance satiety, with a minimal risk of hypoglycaemia.

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